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I, JANENE PEISKER, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2004901726 for a patent by WOMEN'S AND CHILDREN'S HOSPITAL INCORPORATED UNDER THE SOUTH AUSTRALIAN HEALTH COMMISSION ACT as filed on 31 March 2004.

WITNESS my hand this
Eleventh day of April 2005

JANENE PEISKER
TEAM LEADER EXAMINATION
SUPPORT AND SALES

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PROVISIONAL SPECIFICATION FOR AN INVENTION ENTITLED

Invention title: SCREENING FOR LYSOSOMAL STORAGE DISEASE STATUS

Name of Applicant: WOMEN'S AND CHILDREN'S HOSPITAL

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The invention is described in the following statement:

BACKGROUND OF THE INVENTION

Most lysosomal storage disorders (LSD) are inherited in an autosomal recessive manner with the exception of Fabry disease, Danon disease and mucopolysaccharidosis (MPS) type II, which display X-linked recessive inheritance. Some LSD have been classified into clinical subtypes (such as the Hurler/Scheie variants of MPS I, or the infantile/juvenile/adult onset forms of Pompe disease), but it is clear that most LSD have a broad continuum of clinical severity and age of presentation. With the advent of molecular biology/genetics and the characterisation of many of the LSD genes, it is now recognised that the range of severity may, in part, be ascribed to different mutations within the same gene. However, genotype/phenotype correlations do not always hold and other factors including genetic background and environmental factors, presumably play a role in disease progression.

LSD are rare disorders with incidences ranging from about 1:50,000 births to less than 1:4,000,000 births (1). However, when considered as a group, the combined incidence is substantially higher. We have previously estimated the prevalence of LSD in Australia to be 1:7,700 births, excluding the neuronal ceroid lipofuscinoses. The prevalence of this latter group of LSD has been reported to be as high as 1 per 12,500 births in the United States (2). In Finland, incidence values of 1 per 13,000 births for infantile and 1 per 21,000 births for juvenile forms have been reported (3). Clearly, the neuronal ceroid lipofuscinoses will contribute significantly to the overall prevalence of LSD. It is equally certain that additional LSD will be identified as our understanding of lysosomal biology and the clinical manifestations resulting from lysosomal dysfunction improve. A conservative estimate of the prevalence of LSD in the Australian population would be 1 in 5,000 births.

Inborn errors of metabolism causing lysosomal storage have well-recognised effects on neuronal function. In many of the LSD almost all patients develop central nervous system (CNS) dysfunction while in a few disorders such as MPS IVA and MPS VI there are no reports of CNS involvement. In a number of other disorders, notably Gaucher disease, Niemann-Pick disease, MPS I and MPS II, the range of clinical severity spans individuals

with no CNS involvement to those with severe CNS pathology. Notwithstanding the diverse clinical manifestations within LSD, the majority of patients will develop CNS disease.

One of the main determining factors of LSD severity is the residual activity of the affected enzyme. Kinetic models that describe correlations between residual enzyme activity and the turnover rate of its substrate have been proposed (4). Such a mathematical model has been tested in skin fibroblasts and residual activity of β-hexosaminidase A and arylsulphatase A correlated well with substrate turnover (5). However, for many LSD residual enzyme activity is difficult to measure accurately and even when such measurements can be performed they are not always reflective of disease severity, especially CNS pathology. We propose that the level of stored substrates in particular cells or tissues in these disorders, as well as perhaps the levels of secondary metabolites, will reflect disease severity and is likely to yield additional information about the pathophysiology in LSD. The key in determining the absence or presence of CNS pathology lies in understanding the pathogenic process of LSD, which at present is poorly understood.

Unless the context requires otherwise, the word "comprise," or variations such as "comprises" or "comprising" mean the inclusion of a stated element or integer or group of elements or integers, but not the exclusion of any other element or integer or group of elements or integers.

SUMMARY OF THE INVENTION

It has been found that use of estimates of the relative levels of LSD (Lysosomal Storage Disorder) storage associated compounds in body tissues or fluids can be used to assess the LSD status of an individual.

In a first broad form of a first aspect the invention could be said to reside in a method of assessing an LSD status of an individual the method comprising the steps of,

taking a tissue or body fluid sample from the individual,

estimating a level in the sample of each of three or more compound indicators, said indicators being indicative of the level of respectively each of three or more storage associated compounds,

calculating an LSD index number using all of said compound indicators,

and comparing the LSD index number of the sample with a standard to provide an assessment of the LSD status of the individual.

In a first broad form of a second aspect the invention could be said to reside in a method of assessing an LSD status of an individual the method comprising the steps of,

taking a tissue or body fluid sample from the individual,

estimating a level in the sample of each of two or more compound indicators being indicative of the level respectively of each of two or more storage associated compounds,

calculating an LSD index number using all of said compound indicators,

and comparing the LSD index number of the sample with a standard to provide an assessment of the LSD status of the individual,

the two or more storage associated compounds selected to discriminate between an LSD individual from a non-LSD individual with an acceptable confidence level.

In a first broad form of a third aspect the invention could be said to reside in a method for screening for the status of two or more LSDs in an individual,

taking a single tissue or body fluid sample from the individual,

estimating a level in the sample of each three or more compound indicators being indicative of the concentration respectively of each of three or more storage associated compounds,

calculating a first LSD index number using a first set of two or more of said compound indicators and comparing the first LSD index number of the sample with a first control indicator to provide an assessment of the LSD status of the first LSD,

and calculating a second LSD index number using a second set of two or more of said compound indicators and comparing the second LSD index number of the individual with a

second standard to provide an assessment of the LSD status of the second LSD in the individual.

In a first broad form of a fourth form the invention might be said to reside in a method of developing a diagnostic method comprising the steps of

taking a first group of LSD samples one each from a plurality of LSD individuals affected by one type of LSD,

taking a second group of control samples one each from a plurality of control individuals not affected by LSD

the sample being of a tissue or body fluid of the individual

an LSD group of individuals with LSD

interrogating the first group of samples by mass spectrometry for first levels of a plurality of indicators of respective storage associated compounds,

interrogating the second group of samples by mass spectrometry for second levels of the plurality of indicators,

the storage associated compounds selected from the class of compounds consisting of the group glycolipids, phospholipids, ,

comparing the first levels with the second levels

identifying a first group of storage associated compound which are shown as having increased levels of indicators in the first LSD group compared to the control group,

identifying a second group of storage associated compounds which are shows as having decreased levels of indicators in the LSD group compared to the control group,

formulating a combination of two or more of the first and/or second group of indicators by which to calculate and index number whereby to distinguish LSD samples from control samples, and preferably

preparing a standard being a scale of index numbers reflective of the severity of the LSD.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Glycolipid levels in Dried Blood Spots

Box plots showing the relative levels of glucosylceramide (panel A) and lactosylceramide (panel B) in dried blood spots from control (1), Gaucher patients on enzyme therapy (2) and Gaucher patients not on therapy (3). The centre bar shows the median value, the box denotes the 25th and 75th centiles and the upper and lower bars represent the range. Open circles and stars represent outliers and extreme outliers respectively. N= the number of samples in each group.

Figure 2. Glycolipid levels in Dried Blood Spots

Box plots showing the relative levels of ceramide (panel A) and sphingomyelin (panel B) in dried blood spots from control (1), Gaucher patients on enzyme therapy (2) and Gaucher patients not on therapy (3). The centre bar shows the median value, the box denotes the 25th and 75th centiles and the upper and lower bars represent the range. Open circles and stars represent outliers and extreme outliers respectively. N= the number of samples in each group.

Figure 3. Glycolipid Ratios in Dried Blood Spots

Box plots showing the ratios of glucosylceramide to lactosylceramide (panel A) and ceramide to sphingomyelin (panel B) in dried blood spots from control (1), Gaucher patients on enzyme therapy (2) and Gaucher patients not on therapy (3). The centre bar shows the median value, the box denotes the 25th and 75th centiles and the upper and lower bars represent the range. Open circles and stars represent outliers and extreme outliers respectively. N= the number of samples in each group.

Figure 4. Glycolipid Analysis in Dried Blood Spots

Box plots showing the ratio of (glucosylceramide x ceramide) / (lactosylceramide x sphingomyelin) (panel A) and a discriminate function of the same four analytes (panel B) in dried blood spots from control (1), Gaucher patients on enzyme therapy (2) and Gaucher

patients not on therapy (3). The centre bar shows the median value, the box denotes the 25th and 75th centiles and the upper and lower bars represent the range. Open circles and stars represent outliers and extreme outliers respectively. N= the number of samples in each group.

Figure 5. Relative lipid levels in dried blood spots from treated and untreated Gaucher disease patients. Relative glucosylceramide (panel A) and ceramide (panel B) were determined in dried blood spots from patients that were either untreated or had been receiving enzyme replacement therapy for up to 130 months. The shaded area shows the normal range for each analyte.

Figure 6. Relative lipid ratios in dried blood spots from treated and untreated Gaucher disease patients. The ratio of (glucosylceramide x ceramide) / (lactosylceramide x sphingomyelin) (panel A) and a discriminate function of the same four analytes (panel B) were determined in dried blood spots from patients that were either untreated or had been receiving enzyme replacement therapy for up to 130 months. The shaded area shows the normal range for each ratio or function.

Figure 7. Correlation between relative lipid levels in dried blood spots from treated and untreated Gaucher disease patients and chitotriosidase values. Glucosylceramide (panel A) and ceramide (panel B) were determined in dried blood spots from patients that were either untreated or had been receiving enzyme replacement therapy for up to 130 months. The lipid levels were related to the chitotriosidase levels determined in the same patients at the same time.

Figure 8. Correlation between relative lipid ratios in dried blood spots from treated and untreated Gaucher disease patients and chitotriosidase values. Glucosylceramide: lactosylceramide ratio (panel A) and ceramide:sphingomyelin ratio (panel B) were determined in dried blood spots from patients that were either untreated or had been

receiving enzyme replacement therapy for up to 130 months. The lipid levels were related to the chitotriosidase levels determined in the same patients at the same time.

Figure 9. Correlation between relative lipid ratios in dried blood spots from treated and untreated Gaucher disease patients and chitotriosidase values. The ratio of (glucosylceramide x ceramide) / (lactosylceramide x sphingomyelin) (panel A) and a discriminate function of the same four analytes (panel B) were determined in dried blood spots from patients that were either untreated or had been receiving enzyme replacement therapy for up to 130 months. The lipid levels were related to the chitotriosidase levels determined in the same patients at the same time.

Figure 10. Lipid concentrations in urine from controls, Fabry and Fabry heterozygotes. Urine samples (1.5 mL) were extracted with $CHCl_3$ by the method of Bligh/Dyer. Lipids were analysed by tandem mass spectrometry as described previously. The box plots show the median levels of each lipid type (centre bar), the 25th and 75th centiles (boxes) and the upper and lower limits (upper and lower bars). The circles and stars represent outliers and extreme outliers respectively.

Figure 11. Lactosylceramide and trihexosylceramide concentrations in urine from controls, Fabry and Fabry heterozygotes. Urine samples (1.5 mL) were extracted with $CHCl_3$ using the method of Bligh/Dyer. Lipids were analysed by tandem mass spectrometry as described previously. The scatter plots show the relationship between the LC and CTH species. Fabry het (affected) patients were heterozygotes who had been diagnosed with clinical symptoms of Fabry disease; clinical details were not available for the other heterozygotes. Two of the Fabry patients were known to have undergone renal transplants (Fabry (RT)).

Figure 12. Lipid ratios in urine from controls, Fabry and Fabry heterozygotes. Urine samples (1.5 mL) were extracted with $CHCl_3$ using the method of Bligh/Dyer. Lipids were analysed by tandem mass spectrometry as described previously. Each lipid was corrected for the total PC concentration in that sample. The box plots show the median levels of each corrected

lipid type (centre bar), the 25th and 75th centiles (boxes), and the upper and lower limits (upper and lower bars). The circles and stars represent outliers and extreme outliers respectively.

Figure 13. Individual lipid species in urine from controls, Fabry and Fabry heterozygotes. Urine samples (1.5 mL) were extracted with $CHCl_3$ using the method of Bligh/Dyer. Lipids were analysed by tandem mass spectrometry as described previously. Each lipid species was corrected for the total PC concentration in that sample. The box plots show the median levels of each corrected lipid species (centre bar), the 25th and 75th centiles (boxes), and the upper and lower limits (upper and lower bars). The circles and stars represent outliers and extreme outliers respectively.

Figure 14. Selected lipid species concentrations in urine from controls, Fabry and Fabry heterozygotes. Urine samples (1.5 mL) were extracted with $CHCl_3$ using the method of Bligh/Dyer. Lipids were analysed by tandem mass spectrometry as described previously. The scatter plots show the relationship between the lipid species. Fabry het (affected) patients were heterozygotes who had been diagnosed with clinical symptoms of Fabry disease; clinical details were not available for the other heterozygotes. Two of the Fabry patients were known to have undergone renal transplants (Fabry (RT)).

Figure 15. Selected lipids and proteins in urine from controls, Fabry and Fabry heterozygotes. Urine samples (1.5 mL) were extracted with $CHCl_3$ using the method of Bligh/Dyer. Lipids were analysed by tandem mass spectrometry as described previously. The scatter plots show the relationship between the lipid ratios and saposin C. Fabry het (affected) patients were heterozygotes who had been diagnosed with clinical symptoms of Fabry disease; clinical details were not available for the other heterozygotes. Two of the Fabry patients were known to have undergone renal transplants (Fabry (RT)).

Ratio 4 = (LC C24:1*CTH C24:1)/(GC C24:0*SM C24:0) all species corrected for PC.

Figure 16. Individual PC species in urine from controls, Fabry and Fabry heterozygotes. Urine samples (1.5 mL) were extracted with $CHCl_3$ using the method of Bligh/Dyer. Lipids were analysed by tandem mass spectrometry as described previously. Each lipid species was corrected for the total PC concentration in that sample. The box plots show the median levels of each corrected lipid species (centre bar), the 25th and 75th centiles (boxes), and the upper and lower limits (upper and lower bars). The circles and stars represent outliers and extreme outliers respectively.

Figure 17. Lipid concentrations in plasma from controls, Fabry and Fabry heterozygotes. Plasma samples (100 μL) were extracted with $CHCl_3$ using the method of Folsch. Lipids were analysed by tandem mass spectrometry as described previously. The box plots show the median levels of each lipid type (centre bar), the 25th and 75th centiles (boxes), and the upper and lower limits (upper and lower bars). The circles and stars represent outliers and extreme outliers respectively.

Figure 18. Lipid species in plasma from controls, Fabry and Fabry heterozygotes. Plasma samples (100 μL) were extracted with $CHCl_3$ using the method of Folsch. Lipids were analysed by tandem mass spectrometry as described previously. The scatter plots show the relationship between the different lipid species.

Figure 19. Lipid concentrations in whole blood from controls, Fabry and Fabry heterozygotes. Dried blood spots (2 x 3 mm) were extracted with isopropanol and the lipids were analysed by tandem mass spectrometry as described previously. The box plots show the median levels of each lipid type (centre bar), the 25th and 75th centiles (boxes), and the upper and lower limits (upper and lower bars). The circles and stars represent outliers and extreme outliers respectively.

Figure 20. Lipid species in whole blood from controls, Fabry and Fabry heterozygotes. Dried blood spots (2 x 3 mm) were extracted with isopropanol and the lipids were analysed by tandem mass spectrometry as described previously. The box plots show the median levels of each lipid species (centre bar), the 25th and 75th centiles (boxes), and the upper and lower

limits (upper and lower bars). The circles and stars represent outliers and extreme outliers respectively.

Figure 21. CTH species in whole blood from controls, Fabry and Fabry heterozygotes. Dried blood spots (2 x 3 mm) were extracted with isopropanol and the lipids were analysed by tandem mass spectrometry as described previously. The box plots show the median levels of each CTH species (centre bar), the 25th and 75th centiles (boxes), and the upper and lower limits (upper and lower bars). The circles and stars represent outliers and extreme outliers respectively.

Figure 22. Lipid species in whole blood from controls, Fabry and Fabry heterozygotes. Dried blood spots (2 x 3 mm) were extracted with isopropanol and the lipids were analysed by tandem mass spectrometry as described previously. The scatter plots show the relationship between the different lipid species.

DETAILED DESCRIPTION OF THE ILLUSTRATED AND EXEMPLIED EMBODIMENTS OF THE INVENTION

Lysosomes are organelles in eukaryotic cells that function in the degradation of macromolecules, including glycosphingolipids, glycogen, mucopolysaccharides, oligosaccharides, aminoglycans, phospholipids and glycoproteins, into component parts that can be reused in biosynthetic pathways or discharged by cells as waste. The metabolism of exo- and endogenous high molecular weight compounds normally occurs in the lysosomes, and the process is normally regulated in a stepwise process by degradation enzymes. However, when a lysosomal enzyme is not present in the lysosome or does not function properly, the enzymes specific macromolecular substrate accumulates in the lyosome as "storage material" causing a variety of diseases, collectively known as lysosomal storage diseases. In each of these diseases, lysosomes are unable to degrade a specific compound or group of compounds because the enzyme that catalyzes a specific degradation reaction is missing from the lysosome or is present in low concentrations or has been altered.

The field of lysosomal storage disorders is quite active and new LSD are still being found. The present invention is intended to include those that are found from time to time as well as the categories of LSD selected from the group consisting of mucopolysaccharidases (MPSs), lipidoses, glycogenoses, oligosaccharidoses and neruonal ceroid lipofuscinoses. A listing of many of the LSD currently known and the defective enxymes are listed below in table A. It will be understood that the LSD listed therein are encompassed by the present invention.

Table A

Disease	Clinical Phenotype	Enzyme Deficiency
Aspartylglucosaminuria		Aspartylglucosaminidase
Cholesterol ester storage disease	Wolman disease	Acid lipase
Cystinosis		Cystine transporter
Fabry disease	Fabry disease	α-Galactosidase A
Farber Lipogranulomatosis	Farber disease	Acid ceramidase
Fucosidosis		α-L-Fucosidase
Galactosialidosis types I/II		Protective protein
Gaucher disease types I/II/III	Gaucher disease	Glucocerebrosidase (β-glucosidase)
Globoid cell leucodystrophy	Krabbe disease	Galactocerebrosidase
Glycogen storage disease II	Pompe disease	α-Glucosidase
GM1-Gangliosidosis types I/II/III		β-Galactosidase
GM2-Gangliosidosis type I	Tay Sachs disease	β-Hexosaminidase A
GM2-Gangliosidosis type II	Sandhoff disease	β-Hexosaminidase A & B
GM2-Gangliosidosis		GM2-activator deficiency
α-Mannosidosis types I/II		α-D-Mannosidase
β-Mannosidosis		β-D-Mannosidase
Metachromatic leucodystrophy		Arylsulphatase A
Metachromatic leucodystrophy		Saposin B

Mucolipidosis type I	Sialidosis types I/II	Neuramindase
Mucolipidosis types II/III	I-cell disease; pseudo-Hurler polydystrophy	Phosphotransferase
Mucolipidosis type IIIC	pseudo-Hurler polydystrophy	Phosphotransferase γ-subunit
Mucolipidosis type IV		Unknown
Mucopolysaccharidosis type I	Hurler syndrome; Scheie syndrome	α-L-Iduronidase
Mucopolysaccharidosis type II	Hunter syndrome	Iduronate-2-sulphatase
Mucopolysaccharidosis type IIIA	Sanfilippo syndrome	Heparan-N-sulphatase
Mucopolysaccharidosis type IIIB	Sanfilippo syndrome	α-N-Acetylglucosaminidase
Mucopolysaccharidosis type IIIC	Sanfilippo syndrome	AcetylCoA:N-acetyltransferase
Mucopolysaccharidosis type IIID	Sanfilippo syndrome	N-Acetylglucosamine 6-sulphatase
Mucopolysaccharidosis type IVA	Morquio syndrome	Galactose 6-sulphase
Mucopolysaccharidosis type IVB	Morquio syndrome	β-galactosidase
Mucopolysaccharidosis type VI	Maroteaux-Lamy syndrome	N-Acetylgalactosamine 4-sulphatase
Mucopolysaccharidosis type VII	Sly syndrome	β-Glucuronidase
Mucopolysaccharidosis type IX		hyaluronoglucosaminidase-1
Multiple sulphatase deficiency		Multiple sulphatases
Neuronal Ceroid Lipofuscinosis, CLN1	Batten disease	Palmitoyl protein thioesterase

Neuronal Ceroid Lipofuscinosis, CLN2	Batten disease	Tripeptidyl peptidase I
Neuronal Ceroid Lipofuscinosis, CLN3	Vogt-Spielmeyer disease	Unknown
Neuronal Ceroid Lipofuscinosis, CLN5	Batten disease	Unknown
Neuronal Ceroid Lipofuscinosis, CLN8	Northern Epilepsy	Unknown
Niemann-Pick disease types A/B	Niemann-Pick disease	Acid sphyngomyelinase
Niemann-Pick disease type C1	Niemann-Pick disease	Cholesterol trafficking
Niemann-Pick disease type C2	Niemann-Pick disease	Cholesterol trafficking
Pycnodysostosis		Cathepsin K
Schindler disease types I/II	Schindler disease	α-Galactosidase B
Sialic acid storage disease	Sialuria, Salla disease	Sialic acid transporter

The term "storage associated compound" use herein encompasses lipid containing primary storage material that accumulates in lysosomes of cells of the individual with the LSD concerned. The term storage associated compound also encompasses, lipid containing secondary material such as metabolites or catabolite of the primary storage material. The term storage associated material also encompasses lipid containing compounds the concentration of which alters as a consequence of the LSD such as might accumulates as a result of the proliferation of the membrane mass in the cells, or other secondary metabolic compounds that might for example decrease in level as a result of influence exerted by the increasing build up of primary storage material. The term is not intended to encompass the presence or absence of, for example, surface markers, specialised proteins such as enzymes or the like.

The estimated levels might refer directly to the principal storage compound and important candidates are secondary metabolites where these are lipid containing.

In certain forms of the invention the storage compounds might be very wide. They might include lipids and lipid containing macromolecules. The storage associated compounds might thus be selected from the group of compounds consisting of , phospholipids and glycoconjugates

In forms where glycoconjugates are contemplated they might include , , , glycolipids and lipopolysaccharides.

Glycolipids might be selected from the group comprising glycerolipids, glycoposhatidylinositols, glycosphingolipids. The glycosphingolipids might be selected from the group comprising neutral or acidic glycosphingolipids, monoglycosylceramides, or diosylcermaides, gangliosides, glycuronoglycosphingolipids, sulfatoglycosphingolipids, phosphoglycosphingolipids, phosphonoglycosphingolipids, sialoglycosphingolipids, uronoglycosphingolipids, sulfoglycosphingolipids, phosphoglycosphingolipids. Also contemplated may be sphinoglipids (including ceramide, glucosylceramide, trihexosylceramide), and globosides (including tetrahexosylceramides)

The phospholipid useful for the present invention is not intended to be limited. Phospholipids encompassed by the invention might be characterised by their head groups which might be selected from, but not limited to, the group consisting of phosphatidyl serine, phosphatidylinositol, phosphatidyl ethanolamine and sphingomyelin phosphatidyl glycerol, phosphatidyl serine, phosphatidyl inositol, phosophatidyl ethanolamine, cerebroside or a ganglioside

The phospholipids might be characterised by the fatty acids which might be selected from, but not limited to, the group consisting of 1-palmitoyl-2-oleoyl-, 1-palmitoyl-2-linoleoyl -, 1-palmitoly-2-arachadonyl -, 1-palmitoyl-2-docosahexanoyl. However other fatty acyl groups might also be chosen to provide particular characteristics to the spherical rHDL particles and therefore fatty acyl groups might be selected from those having acyl chains of about 12 to

about 18 carbon atoms. These tail group will be understood to be combined with any one of the head groups of the immediately preceding paragraph.

The methods might contemplate utilising markers in addition to the measurement of the levels of storage associated compounds. Such markers may be saponins which might be selected from the group selected from a, b, c and d, or alternatively the markers may comprise the further group consisting of Lamp-1, Lamp-2, Limp-II, mannose-6phosphate receptor, 4-sulphatase, acid phosphatase (ACP), β-hexosaminidase or α mannosidase. The may assist with assigning the status of the individual being screened as either a positive or negative for the LSD(s) being assessed.

The method of measuring the presence and relative levels of storage associated compounds is not important to the general approach of the invention, and might be selected from any convenient method. Such methods might include electrophoresis, chromatography, Gas chromatography, HPLC (High pressure Liquid Chromatography), Nuclear Magnetic resonance analysis, gas chromatography-mass spectrometry (GC-MS), GC linked to Fourier-transform
infrared spectroscopy (FTIR), and silver ion and reversed-phase high-performance liquid chromatography (HPLC) as wells and mass spectrometry

As the complex relationships between stored substrates and pathology in LSD become clearer there is an obvious advantage of providing for faster and more accurate methods to characterise and quantify these stored substrates. That is particularly the case where the storage associated compounds needs to be measured in complex biological samples such as urine, plasma, and blood. To that end it is preferred to use mass spectrometry. The type of mass spectrometry method selected from the group consisting of ionising mass spectrometry, quadrupole mass spectrometry, ion trap mass spectrometry, time-of-flight mass spectrometry and tandem mass spectrometry, and Electrospray ionization (ESI), the later being considered advantageous.

Particularly advantageous is electrospray ionisation-tandem mass spectrometry (ESI-MSMS). The advent of electrospray ionisation-tandem mass spectrometry (ESI-MSMS) has made possible the simultaneous determination of large numbers of analytes from complex mixtures. For newborn screening, ESI MSMS enables the concurrent determination of a wide range of amino acids and acyl carnitines as their butyl esters. This technology is used to screen for over twenty different genetic disorders, including the amino acidopathies and the fatty acid oxidation defects (6,7). ESI-MSMS has been used effectively to investigate stored substrates in a number of LSD and has great potential in the field of this invention.

It has become evident that the levels of a single storage associated compound are not sufficient to give a clear distinction between varying degrees of exposure of an individual to the effects of an LSD. A comparison between at least two markers is require for a quantitative relationship to emerge. The relationship might be additive so that both storage associated compounds increase in the levels in which they are found where the condition is present, and a comparison is made to an internal control. Preferably in devising the method where at least two compounds are selected one from a first group that increase and a second from a second group that decreases in levels. The values are combined mathematically to arrive at an index number. The relative levels of those two compounds leads to an amplification of the differences between LSD affected individuals and the control population. As indicated earlier the severity of the condition and the index number have a direct correlation. Conversely therefore the value of the index number can be compared to a standard to provide a indication of the level of severity of the condition.

It has been found that a difference in index number between individuals that are positive or negative for an LSD condition by use of such combination can be made statistically significant provided an appropriate combination of storage associated compounds is used.

Samples for analysis can be obtained from any organ, tissue, fluid or other biological sample comprising lysosomes or their component storage associated compounds. A preferred sample is whole blood and products derived therefrom, such as plasma and serum. Blood

samples may conveniently be obtained from blood-spot taken from, for example, a guthrie card.

Other sources of tissue for example are skin, hair, urine, oral fluids, semen, faeces, sweat, milk, amniotic fluid, liver, heart, muscle, kidney, brain and other body organs. Tissue samples comprising whole cells are typically lysed to release the storage associated compounds.

The present method may be used as an early test and thus samples can be obtained from embryos, foetuses, neonatals, young infants.

Most preferably the sample is one readily obtainable such as a blood samples. Whilst obtaining these is invasive they are routinely taken and generally therefore are not inconvenient. It may be preferred to have a non-invasive sample such as urine, oral fluid or buccal smear. There are however variations in the value of certain metabolites in urine resulting from variation in salt content, such as oxalic acid, and in saliva there is variation in the capacity of individuals to secrete certain compounds.

It is found that with Gaucher patients that the LSD index number was not only a qualitative measure but also a qualitative measure being indicative of the severity of the condition. Thus the status of the LSD being assessed may not only be to ascertain the presence or absence but might also include the degree of severity. The status might also include subclinical levels of the condition that relate to levels achieved before onset of physical manifestations become apparent. This invention will be understood to have application to monitoring treatment, for example with individuals undergoing enzyme or other therapy.

Thus individuals with Gaucher disease that undergo enzyme replacement therapy have a index number that is considerably lower than untreated individuals. It is also desirable that the doses of active enzyme delivered to sufferers is kept to a minimum if only from a cost perspective but perhaps also from a perspective of minimising any adverse affects of the

treatment. Thus the present method may be used particularly for monitoring treatment of an LSD sufferer, or for ascertaining initially and perhaps from time to time as the sufferer ages the most appropriate dose of active to be delivered, and thus individuals diagnosed may be tested from time to time to ascertain the severity of the condition. It is less critical that the test discriminates quite as distinctly from non-LSD sufferers because all that is required is that the relative level of severity can be quantified. Thus whilst it may be necessary to screen using indicators of the concentration of three or more lipid containing compounds to distinguish over non-LSD sufferers the monitoring may only require indicators of two lipid containing compounds and may be carried out using less precise measuring methods.

The invention has particular applicability to human conditions. Certain mammals are also susceptible to LSD and the invention may be useful where the individual is a non-human mammal. For examples α-mannosidoses is relatively common in certain breeds of cattle and screening may be a useful stock management tool.

EXAMPLE 1 MONITORING OF THERAPY FOR GAUCHER DISEASE

This report provides a detailed analysis of the initial trial of our developed methodology to monitor enzyme replacement therapy (ERT) in Gaucher disease using dried blood spots.

Patient samples: Dried blood spots have been collected from five Australian Gaucher patients receiving ERT for the past two years (12 samples). Sixteen dried blood spots have been collected from patients not receiving ERT, from referrals to the National Referral Laboratory for Lysosomal, Peroxisomal and Related Diseases (which is based in our parent Department). In addition, through collaboration with Dr Eugene Mengel (Germany), we have obtained 39 samples from German Gaucher disease patients receiving ERT, and three samples from untreated patients. Dried blood spots have been collected from 10 unaffected adults as control samples. Total sample numbers are as shown in Table 1.

Sample preparation: From each Guthrie card sample a 3 mm dried blood spot was punched and the lipids were eluted (16h) with 200 μL of isopropanol containing 200 nmol of each

internal standard; Cer C17:0, GC(*d3*)C16:0, LC(*d3*)C16:0, PC C14:0. The blood spots were removed and the isopropanol dried under a stream of nitrogen. Lipids were redissolved in 100 µL of methanol containing 10 mM NH_4COOH for analysis by mass spectrometry.

Mass spectrometry: Mass spectrometric analysis of lipids was performed using a PE Sciex API 3000 triple-quadrupole mass spectrometer with a turbo-ionspray source and Analyst data system (PE Sciex, Concord, Ontario, Canada). Samples (20 µL) were injected into the electrospray source with a Gilson 233 autosampler using a carrying solvent of methanol at a flow rate of 80 µL/minute. For all analytes nitrogen was used as the collision gas at a pressure 2×10^{-5} Torr. Lipids were analysed in +ve ion mode. Determination of lipids was performed using the multiple-reaction monitoring (MRM) mode. Seventeen different glycosphingolipid and ceramide species were monitored using the ion pairs shown in Table 2. Each ion pair was monitored for 100 milliseconds and the measurements were repeated and averaged over the injection period. Determination of lipids was achieved by relating the peak height of each lipid ion signal to the peak height of the signal from the corresponding internal standard (Table 2).

Results: To determine which analytes were potentially useful markers for monitoring Gaucher disease, the patients were grouped into control (group 1, n=10), Gaucher patients receiving ERT (group 2, n=51), and untreated Gaucher patients (group 3, n=19). Mann-Whitney U values were then calculated for each analyte to determine the difference between the control and untreated patients, control and treated patients, and treated and untreated patients. These results are shown in Table 3.

We observed that, in addition to the expected elevation of glucosylceramide (GC) in the untreated Gaucher patients compared to controls, there were significant differences in the level of ceramide C16:0 and the sphingomyelin species C16:0, C22:0 and C24:0 (all significant to the 0.01 level). The same markers also showed a significant difference between treated and untreated Gaucher patients. Of the lactosylceramide and

trihexosylceramide species only the C16:0 species showed a significant difference between control and untreated patients (significant to the 0.05 level). The box plots of each C16:0 species of ceramide, GC, LC and sphingomyelin (Figures 1 and 2) show that whilst there is an observed increase in the level of ceramide and GC in untreated patients, the levels of sphingomyelin and LC are decreased. In addition, the level of these analytes in the treated patients generally fell between the control and untreated patients. In each case ERT has partially normalised the lipid levels, although not in all patients.

Although the observed differences between control and untreated patients are significant there is still considerable overlap between the two populations. This is due, at least in part, to the range of lipid levels in the control and patient groups. To improve the discrimination of the markers we investigated the use of multiple markers by plotting ratios of GC/LC or ceramide/sphingomyelin (Figure 3). As GC and ceramide levels increase in Gaucher patients, while the LC and sphingomyelin decrease, these ratios provided improved discrimination between groups. Utilising all four analytes in a combined ratio (Ratio4 = (GC C16:0*Cer C16:0)/(LC C16:0*SM C16:0) further improved the discrimination. Similarly discriminate analysis using the four C16:0 species resulted in a function (Dis2 = (-195*Cer C16:0) - (29.8*GC C16:0) + (12.3*LC C16:0) + (16.9*SM C16:0) - 1.91)) with improved discrimination. (Figure 4 and Table 3).

Clearly, the use of multiple analytes or lipid profiles provides a better representation of lipid metabolism in control and Gaucher patients. The ratio4 and discriminate function (Dis2) plotted in Figure 4 show almost total separation of the control and untreated Gaucher patient groups, with the patient group being partially normalised (although many treated patients were not completely normalised)

We investigated what effect time on therapy had on a number of the same analytes and analyte ratios (Figure 5 and 6). The GC and ceramide levels showed a trend towards normalisation with increasing time on therapy, however in a number of patients the ceramide level did not reach the normal range even at 80-120 months on therapy. The use of the ratio

and the discriminate function (Figure 6) showed similar results with some patients normalising with time but others outside the normal range even after 80-120 months of therapy.

The relationship between the glycolipid markers and ratios, and the macrophage activation marker chitotriosidase is shown in Figures 7-9; a significant correlation is observed for the ceramide and GC as well as for the ratios GC/LC, ceramide/sphingomyelin and ratio4, and for the discriminate function. Table 4 shows the Pearson correlation coefficients for these markers with chitotriosidase and other markers that have been used to monitor ERT in Gaucher disease including angiotensin converting enzyme, lysozyme and acid phosphatase. In general the correlations are stronger between these markers and the lipid ratios, rather than single lipid species.

Discussion: In this study we have provided evidence that the primary storage substrate GC is a useful marker for monitoring Gaucher disease. We observe an increased level of GC in dried blood spots from untreated patients compared to controls and a normalisation of GC levels after ERT. This is an expected outcome, based on the known biochemistry of Gaucher disease. Somewhat less expected is the elevation in ceramide and the decrease in LC and sphingomyelin. We have previously reported that LC is decreased in the plasma of Gaucher patients and that the ratio of GC/LC provides a better discrimination of Gaucher patients from controls than the GC levels on their own (Whitfield et al 2002). In these preliminary studies we have identified that other lipids are also affected, particularly ceramide and sphingomyelin. We have also shown that using a combination of these analytes with the GC and LC levels, as either a ratio or a discriminate function, provides greater discrimination and potentially a better mechanism for monitoring ERT in Gaucher disease than the use of individual analytes. The ratio4 and the discriminate function Dis2 are based on the limited numbers in this study and require further refinement, however they provide an initial demonstration of the power of metabolic profiling for the characterisation of patients and the monitoring of therapy in Gaucher disease.

Our hypothesis is that the level of GC within a normal population will fall within a specified range, which is affected by many metabolic parameters affecting the biosynthesis and degradation of GC. In the Gaucher disease population this range will be altered as a result of the metabolic defect; however, those Gaucher patients with the lower GC levels are likely to overlap with unaffected controls with the higher GC levels. This results in uncertainties in the interpretation of GC levels in isolation with regard to Gaucher disease status, and difficulties in determining normalisation following ERT.

However, with ametabolic profile (multiple analytes) the breadth of the normal range will be decreased, as each of these analytes is related to the others by the metabolic pathways that exist. Consequently, the power to discriminate normal from Gaucher disease is increased and the ability to measure the normalisation of patients on treatment is improved.

Table 1. Patient and control samples included in this trial

Patient group	Number	Age Median (range)	Comment
Control	10	38 (23-56)	
Treated Gaucher	51	23 (2-72)	All type 1
Untreated Gaucher	19	24 (1-36)	2 type 3, 14 type 1, 3 unknown

Table 2. Lipid analytes used for Gaucher Monitoring

Lipid analytes[a]	Internal standard	MRM ion pairs (*m/z*)
Cer C16:0	Cer C17:0	538.7/264.4
Cer C24:0	Cer C17:0	650.7/264.4
Cer C24:1	Cer C17:0	648.7/264.4
Cer C17:0 (internal standard)		552.7/264.4
GC C16:0	GC(*d3*)C16:0	700.6/264.4
GC C22:0	GC(*d3*)C16:0	784.7/264.4
GC C24:0	GC(*d3*)C16:0	812.7/264.4
GC C24:1	GC(*d3*)C16:0	810.8/264.4
GC(*d3*)C16:0 (internal standard)		703.8/264.4
LC C16:0	LC(*d3*)C16:0	862.4/264.4
LC C24:0	LC(*d3*)C16:0	974.8/264.4
LC C24:1	LC(*d3*)C16:0	972.8/264.4
CTH C16:0	LC(*d3*)C16:0	1024.1/264.4
CTH C22:0	LC(*d3*)C16:0	1108.1/264.4
CTH C24:0	LC(*d3*)C16:0	1136.6/264.4
CTH C24:1	LC(*d3*)C16:0	1134.1/264.4
LC(*d3*)C16:0 (internal standard)		865.6/264.4
SM C16:0	PC C14:0	703.9/184.1
SM C22:0	PC C14:0	787.8/184.1
SM C24:0	PC C14:0	815.8/184.1
PC C14:0 (internal standard)		678.5/184.1

[a] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihoxoside, SM = sphingomyelin, PC = phosphatidylcholine

5

Table 3. Mann-Whitney U values for lipid analytes and ratios of analytes, between controls[a], untreated Gaucher patients[b] and Gaucher patients treated with enzyme replacement therapy[c].

	Control vs Untreated		Control vs Treated		Untreated vs Treated	
Analyte	M-W U[d]	Sig.[e]	M-W U[d]	Sig.[e]	M-W U[d]	Sig.[e]
Cer C16:0	6	0.000	111	0.004	300	0.009
Cer C24:1	73	0.313	215	0.342	478	0.740
Cer C24:0	56	0.070	174	0.087	447	0.466
GC C16:0	9	0.000	139	0.017	240	0.001
GC C22:0	26	0.002	142	0.021	307	0.012
GC C24:1	19	0.000	101	0.002	271	0.003
GC C24:0	28	0.002	149	0.029	319	0.018
LC C16:0	49	0.033	222	0.419	358	0.063
LC C24:0	75	0.359	183	0.121	450	0.490
LC C24:1	62	0.130	228	0.481	434	0.375
CTH C16:0	52	0.046	149	0.028	392	0.152
CTH C22:0	83	0.582	127	0.009	166	0.000
CTH C24:1	88	0.748	103	0.002	189	0.000
CTH C24:0	54	0.060	179	0.104	472	0.687
SM C16:0	31	0.003	239	0.618	149	0.000
SM C22:0	29	0.002	203	0.240	187	0.000
SM C24:0	33	0.004	219	0.382	219	0.000
GC_LC	6	0.000	80	0.001	169	0.000
CER_SM	9	0.000	150	0.031	138	0.000
RATIO4[f]	7	0.000	64	0.000	96	0.000
DIS2[g]	9	0.000	164	0.057	86	0.000

[a] controls n=10

[b] untreated n= 19

[c] treated n= 51

[d] Mann-Whitney U values

[e] significance (two-tailed)

[f] Ratio4 = (GC C16:0*Cer C16:0)/(LC C16:0*SM C16:0)

[g] Dis2 = (-195*Cer C16:0) - (29.8*GC C16:0) + (12.3*LC C16:0) + (16.9*SM C16:0) - 1.91

Table 4. Pearson Correlation coefficients between lipid markers and other markers used in Gaucher disease.

Analyte[a]	months of therapy[b]		chitotriosidase(nmol/ml/h)		ACE (U/l)[c]		lysozyme (mg/l)		acid phosphatase	
	PCC[c] N=51	Sig.[d]	PCC N=30	Sig.	PCC N=40	Sig.	PCC N=38	Sig.	PCC N=40	Sig.
Cer C16:0	-0.24	0.08	0.40	0.03	0.42	0.01	0.40	0.01	0.44	0.00
GC C16:0	-0.32	0.02	0.41	0.02	0.36	0.02	0.23	0.17	0.52	0.00
LC C16:0	0.19	0.18	0.16	0.38	0.10	0.53	0.01	0.96	0.17	0.30
CTH C16:0	0.00	1.00	-0.10	0.60	-0.03	0.83	0.34	0.04	-0.01	0.95
SM C16:0	0.51	0.00	-0.29	0.13	-0.24	0.13	0.04	0.82	-0.23	0.15
GC/LC	-0.35	0.01	0.42	0.02	0.41	0.01	0.23	0.17	0.50	0.00
CER/SM	-0.47	0.00	0.52	0.00	0.50	0.00	0.35	0.03	0.53	0.00
RATIO4	-0.38	0.01	0.59	0.00	0.58	0.00	0.39	0.01	0.70	0.00
DIS2	0.54	0.00	-0.49	0.01	-0.47	0.00	-0.26	0.11	-0.47	0.00

[a] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihexoside, SM = sphingomyelin, Ratio4 = (GC C16:0*Cer C16:0)/(LC C16:0*SM C16:0), Dis = (-195*Cer C16:0) - (29.8*GC C16:0) + (12.3*LC C16:0) + (16.9*SM C16:0) - 1.91

[b] months on enzyme replacement therapy

[c] PCC = Pearson correlation coefficient

[d] Sig. = significance (two tailed)

[e] ACE = angiotensin converting enzyme

EXAMPLE 2.

IDENTIFICATION OF FABRY HEMIZYGOUS AND HETEROZYGOUS INDIVIDUALS USING LIPID PROFILES.

This report summarises the results of analyses performed on urine, plasma and dried blood spots from control, Fabry heterozygote and Fabry patient groups.

MATERIALS AND METHODS

Patient samples: Urine samples have been collected from 14 Fabry patients (two of whom had renal transplants), 13 Fabry heterozygotes (three of whom had reported clinical symptoms) and 20 unaffected controls. Plasma samples were retrieved from archival sources in the Department of Chemical Pathology and represented 29 Fabry patients, three Fabry heterozygotes and 10 control samples. Dried blood spots on filter paper (Guthrie cards) were collected from 13 Fabry patients, two Fabry heterozygotes and 10 control individuals.

Sample preparation and analysis: Urine, plasma and dried blood spot samples were prepared as described in Appendices I, II and III, and analysed for lipids by mass spectrometry.

Mass spectrometry: Mass spectrometric analysis of lipids was performed using a PE Sciex API 3000 triple-quadrupole mass spectrometer with a turbo-ionspray source and Analyst data system (PE Sciex, Concord, Ontario, Canada). Samples (20 µL) were injected into the electrospray source with a Gilson 233 autosampler using a carrying solvent of methanol at a flow rate of 80 µL/minute. For all analytes nitrogen was used as the collision gas at a pressure 2×10^{-5} Torr. Lipids were analysed in +ve ion mode. Lipid analysis was performed using the multiple-reaction monitoring (MRM) mode. Twenty-two different ceramide, glycosphingolipid and sphingomyelin species were monitored using the ion pairs shown in Table 5. In urine samples seven additional phosphatidylcholine species were also monitored (Table 5). Each ion pair was monitored for 100 milliseconds and the measurements were repeated and averaged over the injection period. Determination of lipids was achieved by relating the peak height of each lipid ion signal to the peak height of the signal from the corresponding internal standard (Table 5).

RESULTS

Analysis of Urine: Lipid profiling of the urine samples from control, Fabry and Fabry heterozygotes (Fabry het) has been performed. In all, 29 lipid species were determined including ceramide (Cer), glucosylceramide (GC), lactosylceramide (LC), trihexosylceramide (CTH), sphingomyelin (SM) and phosphatidylcholine (PC) species. Appropriate internal standards were used that provide absolute quantification of these species (expressed as nmol/L urine). PC was included as a general marker of urinary sediment, as we had previously observed this to be a more useful correction factor for the determination of urinary lipids than creatinine. This relates to the urinary lipids being derived from epithelial cells of the kidneys, bladder and urinary tract rather than filtered through the kidneys; PC is a major lipid constituent of these cells and so is a useful measure of the level of urinary sediment.

An initial statistical analysis was performed on the data as expressed as nmol/L urine. Mann-Whitney U values were determined to compare the control group with the Fabry and Fabry het groups (Table 6). Examination of these results shows that many of the lipid analytes are significantly different in the patient groups compared to the control groups. The Fabry and Fabry het groups show a significant difference to the control group in many lipid species, including Cer, LC, CTH and SM. Interestingly, the level of PC in the Fabry het group is significantly elevated above the control population, while no significant difference between the control and Fabry groups is observed. Examination of the range of analytes for each group (Figure 10) shows that for all analytes except CTH, the Fabry het group is elevated above the control and Fabry groups. The observed elevation of these lipids suggests that the Fabry het group has elevated urinary sediment compared to the control and Fabry groups.

The scatter plot of LC (total) versus CTH (total) (Figure 11A) shows that the use of lipid levels (nmol/L urine) can differentiate between Fabry patients and the control group, although there is some overlap between both Fabry and Fabry het and the control group. The

use of the specific lipid species LC C24:1 and CTH C24:1 (Figure 11B) improved this discrimination, although some overlap still exists. A concern with these results is that the differentiation of the Fabry het group from the control group reflects the elevated urinary sediment rather than an altered lipid profile. Consequently, individuals who are not affected by Fabry disease but who have an elevation in urinary sediment would be falsely identified as a Fabry het using this type of analysis.

To address this, correction was made for each lipid analyte value for the level of PC (total) in each sample; statistical analysis on these data was performed. Table 7 shows the Mann-Whitney U values for each patient group compared to the control group. The corrected data also show multiple analytes to be significantly different between the control and patient groups. The box plots in Figure 12 show the range of each analyte group (corrected for PC). These plots show that the Fabry group has elevated CTH, LC and Cer and decreased SM, whereas the Fabry het group now shows an elevation in CTH and a much lower elevation in LC and Cer. Interestingly, the Fabry het group shows a larger decrease in the SM than the Fabry group. This may relate to a sex difference, although no difference was seen between the males and females in the control group. Larger sample numbers will be required to confirm this.

As with the urine data expressed as nmol/L the differentiation between control and patient groups could be improved by the selection of specific lipid species. The increases observed in Cer, LC and CTH were greatest in the C24:1 species, and the decreases observed in GC and SM were greatest in the C24:0 species (Figure 13). Following these observations we looked at the relationship between these lipid species in a series of scatter plots and how these were able to differentiate the control and patient groups (Figure 14). Using different combinations we can achieve almost total differentiation between the control and patient groups, particularly with CTH C24:1 and LC C24:1 plotted as a function of SM C24:0 (Figures 5D and 5E).

LC and CTH are elevated while GC and SM are decreased in the patient groups. The use of ratios of these analytes enables further discrimination between the control and patient groups. Figure 15 shows total separation of both Fabry and Fabry het groups from the control group.

Of interest is the observation that the composition of individual PC species is significantly altered in the Fabry group compared to the control group. Some PC species show a proportional elevation (C34:2 and C36:4) while others show a corresponding decrease (C32:1 and C34:1) (Figure 16). On first examination there appears to be a trend toward higher levels of unsaturated fatty acids in the Fabry group. This is supported by the observation that the LC C24:1 and CTH C24:1 species show a greater elevation in the Fabry group compared to the C24:0 species. The effect of these changes in the lipid composition to the cellular function in Fabry disease and the relationship to the pathophysiology of this disorder is unclear at this time. However, we are further investigating these effects in cultured skin fibroblasts from control and Fabry patients. Results will be available in subsequent Reports.

To summarise, analysis of the lipid profile in urine from control, Fabry and Fabry het groups has identified the specific lipid species, ratios and profiles that best discriminate between the control and patient groups. Correction of the lipid species for PC content of the urine improved the discrimination between control and Fabry groups and minimised the potential for the false identification of individuals with high urinary sediment as Fabry hets. The "Ratio 4" (LC C24:1*CTH C24:1)/(GC C24:0*SM C24:0) provides total discrimination of all Fabry and Fabry hets from the control group.

Analysis of Plasma: The number of plasma and blood spot samples available from the Fabry het group were fewer than the urine samples. However, lipid profiles were performed on these samples and the Mann-Whitney U values for each lipid species are shown in Table 8. No significant difference is observed between the control and Fabry het groups (possibly due to the low number of Fabry het samples), however Cer, LC, CTH and SM species show significant differences between the control and Fabry groups. Figure 17 shows that Cer, LC

and SM are decreased in the Fabry group compared to the control group, while CTH is increased and GC is unchanged, although it did appear to have a broader range in the Fabry group. When the Cer, GC, LC and SM C16:0 species were plotted as a function of the CTH C16:0 (Figure 18) a strong correlation is observed in the Fabry group, which provides improved discrimination between the control and Fabry groups.

Analysis of Whole Blood: Analysis of dried blood spots for lipids show relatively few analytes with significant differences between the control and Fabry groups (Table 9). Box plots of the lipid groups (Figure 19) show only slight elevations or decreases in the Fabry compared to the control groups, and only the CTH has a p value of less than 0.05. The use of specific lipid species offers little improvement although the decrease of Cer C24:1 in the Fabry group compared to the control group is significant ($p = 0.03$) (Figure 20). The box plots of the CTH species show that only the C16:0, C18:0 and C20:0 species are significantly different from the control group (Figure 21 and Table 9). The scatter plot of CTH C16:0 as a function of Cer C16:0 (Figure 22A) shows a similar correlation between these two analytes, as was observed in the plasma samples. The correlation is not as pronounced in the plot of CTH C18:0 as a function of SM C16:0 (Figure 22B). The Fabry het group did not show any significant difference to the control group in the lipid analytes.

DISCUSSION

The use of a urinary lipid profile also has potential to identify Fabry and Fabry heterozygotes. While the determination of CTH on its own did not identify all patients, the use of ratios of lipid species provided total discrimination of both the Fabry patients (even after renal transplant) and the heterozygotes from the control group. Urine analysis is a practical, non-invasive procedure to screen large populations at high risk for Fabry disease.

Monitoring of therapy: Characterisation of the lipid profile of Fabry patients in plasma, dried blood spots and urine has highlighted a number of previously unreported differences between Fabry patients and the control population. This technology enables us to very accurately describe the lipid profile from the control population and so define how the profile differs in Fabry disease. Significant differences were observed in most lipid groups

suggesting that Fabry disease results in a general alteration of lipid metabolism, not just the storage of trihexosylceramide. With further validation it will be possible to monitor therapy in Fabry disease by following the total lipid profile as it is corrected from the disease state to a normal profile. This will provide a more comprehensive Fabry monitoring program than current methods allow. We are currently investigating the potential of this approach with patient samples and cultured skin fibroblasts.

Prediction of disease severity: The detailed description of the disease state provided by the lipid profile described in this Report will significantly improve our ability to describe the disease in any given individual. Correlation of these profiles with known phenotypes and disease progression will enable us to predict disease progression.

Table 5. Lipid analytes used for lipid analysis of Fabry samples

Lipid analytes[a]	Internal standard	MRM ion pairs (*m/z*)
Cer C16:0	Cer C17:0	538.7/264.4
Cer C24:0	Cer C17:0	650.7/264.4
Cer C24:1	Cer C17:0	648.7/264.4
Cer C17:0 (internal standard)		552.7/264.4
GC C16:0	GC(*d3*)C16:0	700.6/264.4
GC C22:0	GC(*d3*)C16:0	784.7/264.4
GC C24:0	GC(*d3*)C16:0	812.7/264.4
GC C24:1	GC(*d3*)C16:0	810.8/264.4
GC(*d3*)C16:0 (internal standard)		703.8/264.4
LC C16:0	LC(*d3*)C16:0	862.4/264.4
LC C20:0	LC(*d3*)C16:0	918.6/264.4
LCC22:0	LC(*d3*)C16:0	946.7/264.4
LC C22:0-OH	LC(*d3*)C16:0	962.7/264.4
LC C24:0	LC(*d3*)C16:0	974.8/264.4
LC C24:1	LC(*d3*)C16:0	972.8/264.4
LC(*d3*)C16:0 (internal standard)		865.6/264.4
CTH C16:0	CTH C17:0	1024.1/264.4
CTH C18:0	CTH C17:0	1052.1/264.4
CTH C20:0	CTH C17:0	1080.1/264.4
CTH C22:0	CTH C17:0	1108.1/264.4
CTH C24:0	CTH C17:0	1136.6/264.4
CTH C24:1	CTH C17:0	1134.1/264.4
CTH C17:0 (internal standard)		1038.1/264.4
SM C16:0	PC C14:0	703.9/184.1
SM C22:0	PC C14:0	787.8/184.1
SM C24:0	PC C14:0	815.8/184.1
PC C32:0	PC C14:0	706.5/184.1
PC C32:1	PC C14:0	704.5/184.1
PC C34:1	PC C14:0	732.5/184.1
PC C34:2	PC C14:0	730.5/184.1
PC 36:2	PC C14:0	758.6/184.1
PC C36:4	PC C14:0	754.6/184.1
PC C38:4	PC C14:0	782.6/184.1
PC C14:0[b] (internal standard)		678.5/184.1

[a] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihoxoside, SM = sphingomyelin, PC = phosphatidylcholine

[b] PC C14:0 is a commercial standard and is known to have a C16:0 second fatty acid (equivalent to PC C30:0)

Table 6 Mann-Whitney U values for lipid[a] analytes in urine.

Analyte[b]	Control (n=20) vs Heterozygote (n=13)		Control (n=20) vs Fabry (n=14)	
	MW-U	p value	MW-U	p value
Cer C16:0	41	0.000	81	0.018
Cer C24:0	82	0.037	118	0.243
Cer C24:1	62	0.006	68	0.005
GC C16:0	63	0.006	144	0.746
GC C22:0	87	0.056	119	0.256
GC C24:0	69	0.012	118	0.243
GC C24:1	71	0.014	153	0.974
LC C16:0	18	0.000	61	0.003
LC C20:0	41	0.000	56	0.001
LCC22:0	34	0.000	77	0.012
LC C22:0-OH	37	0.000	81	0.018
LC C24:0	23	0.000	17	0.000
LC C24:1	11	0.000	2	0.000
CTH C16:0	3	0.000	56	0.001
CTH C18:0	61	0.005	46	0.000
CTH C20:0	22	0.000	59	0.001
CTH C22:0	2	0.000	43	0.000
CTH C24:0	4	0.000	37	0.000
CTH C24:1	0	0.000	25	0.000
SM C16:0	80	0.031	115	0.206
SM C22:0	83	0.041	74	0.009
SM C24:0	120	0.432	70	0.006
PC C32:0	50	0.001	146	0.795
PC C32:1	56	0.003	94	0.052
PC C34:1	65	0.008	129	0.417
PC C34:2	63	0.006	109	0.144
PC 36:2	61	0.005	148	0.846
PC C36:4	64	0.007	103	0.098
PC C38:4	74	0.018	126	0.364
Cer (total)	56	0.003	109	0.083
GC (total)	64	0.007	137	0.386
LC (total)	14	0.000	40	0.000
CTH (total)	0	0.000	37	0.000
SM (total)	85	0.048	84	0.012
PC (total)	62	0.006	164	0.975

[a] lipids expressed as nmol/L urine.

[b] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = trihexosylceramide, SM = sphingomyelin, PC = phosphatidylcholine.

Table 7 Mann-Whitney U values for lipid[a] analytes in urine.

(Corrected for phospatidylcholine content)

Analyte[b]	Control (n=20) vs Heterozygote (n=13)		Control (n=20) vs Fabry (n=14)	
	MW-U	p value	MW-U	p value
Cer C16:0	95	0.101	73	0.009
Cer C24:0	90	0.070	107	0.127
Cer C24:1	141	0.946	60	0.002
GC C16:0	133	0.733	152	0.948
GC C22:0	62	0.006	109	0.144
GC C24:0	63	0.006	119	0.256
GC C24:1	89	0.065	148	0.846
LC C16:0	37	0.000	63	0.003
LC C20:0	128	0.609	69	0.006
LCC22:0	107	0.219	80	0.016
LC C22:0-OH	125	0.539	71	0.007
LC C24:0	62	0.006	2	0.000
LC C24:1	34	0.000	2	0.000
CTH C16:0	87	0.056	35	0.000
CTH C18:0	126	0.562	33	0.000
CTH C20:0	128	0.609	35	0.000
CTH C22:0	68	0.010	26	0.000
CTH C24:0	78	0.026	11	0.000
CTH C24:1	43	0.001	4	0.000
SM C16:0	42	0.001	70	0.006
SM C22:0	47	0.001	0	0.000
SM C24:0	43	0.001	4	0.000
PC C32:0	120	0.432	83	0.021
PC C32:1	136	0.811	28	0.000
PC C34:1	72	0.015	39	0.000
PC C34:2	143	1.000	31	0.000
PC 36:2	84	0.044	135	0.538
PC C36:4	127	0.585	20	0.000
PC C38:4	75	0.020	93	0.048
Cer (total)	119	0.413	82	0.019
GC (total)	83	0.041	129	0.417

LC (total)	77	0.024	26	0.000
CTH (total)	97	0.116	19	0.000
SM (total)	42	0.001	12	0.000

[a] lipids expressed as nmol/umol PC.

[b] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = trihexosylceramide, SM = sphingomyelin, PC = phosphatidylcholine.

Table 8. Mann-Whitney U values for lipid[a] analytes in plasma.

Analyte[b]	Control (n=10) vs Heterozygote (n=2)		Control (n=10) vs Fabry (n=29)	
	MW-U	p value	MW-U	p value
Cer C16:0	9	0.830	59	0.007
Cer C24:1	7	0.519	34	0.000
Cer C24:0	9	0.830	48	0.002
GC C16:0	9	0.830	136.5	0.908
GC C22:0	9	0.830	134	0.842
GC C24:1	6	0.390	137.5	0.934
GC C24:0	2	0.085	124	0.596
LC C16:0	9	0.830	66	0.014
LC C24:1	8	0.667	33	0.000
LC C24:0	4	0.197	4.5	0.000
CTH C16:0	8	0.667	33	0.000
CTH C18:0	7	0.519	19.5	0.000
CTH C20:0	9	0.830	49	0.003
CTH C22:0	4	0.197	45	0.002
CTH C24:1	10	1.000	49	0.003
CTH C24:0	8	0.667	53	0.004
SM C16:0	10	1.000	33	0.000
SM C22:0	4	0.197	39	0.001
SM C24:0	8	0.667	29	0.000
Cer (total)	7	0.519	38.5	0.001
GC (total)	5	0.282	138	0.947
LC (total)	8	0.667	48	0.002
CTH (total)	10	1.000	38	0.001
SM (total)	8	0.667	37	0.001

[a] lipids were calculated as umol/L plasma.

[b] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = trihexosylceramide, SM = sphingomyelin.

Table 9: Mann-Whitney U values for lipid[a] analytes in whole blood.

Analyte[b]	Control (n=10) vs Heterozygote (n=2)		Control (n=10) vs Fabry (n=13)	
	MW-U	p value	MW-U	p value
Cer C16:0	8	0.235	48	0.292
Cer C24:1	8	0.237	30	0.030
Cer C24:0	12	0.612	46.5	0.251
GC C16:0	14	0.866	39	0.107
GC C22:0	7.5	0.202	40.5	0.128
GC C24:1	15	1.000	47.5	0.278
GC C24:0	9	0.310	38.5	0.100
LC C16:0	13	0.735	37.5	0.088
LC C24:1	7	0.175	61.5	0.828
LC C24:0	12	0.612	40.5	0.129
CTH C16:0	10	0.398	6	0.000
CTH C18:0	8	0.237	42.5	0.163
CTH C20:0	9	0.310	45	0.215
CTH C22:0	10	0.398	40	0.121
CTH C24:1	7.5	0.204	1.5	0.000
CTH C24:0	6	0.128	32	0.041
SM C16:0	7.5	0.204	53.5	0.475
SM C22:0	9	0.310	61	0.804
SM C24:0	11	0.499	55.5	0.556
Cer (total)	9	0.310	38	0.094
GC (total)	13	0.735	37	0.082
LC (total)	11	0.499	42	0.154
CTH (total)	9	0.310	23	0.009
SM (total)	12	0.612	63.5	0.926

[a] lipids were calculated as umol/L plasma.

[b] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = trihexosylceramide, SM = sphingomyelin.

APPENDIX I: Procedure for sphingolipid extraction from urine (Bligh-Dyer method).

1. To 1.5 mL urine add 5.6 mL $CHCl_3$/MeOH (1:2)
2. Add 400 pmol internal standards to each sample; 2 µL (d3) C16:0 LC (200 µM); 2 µL (d3) C16:0 GC (200 µM), and 2 µL GM2 (200 µM), 6.25 µL CTH C17:0 (64 µM); 2 µL Cer C17:0 (200 µM), 2 µL PC (200 µM).
3. Place tubes on platform shaker for 10 minutes at 150 opm. Stand tubes at room temperature for at least 50 minutes.
4. Partition with the addition of 1.9 mL $CHCl_3$ and 1.9 mL milliQ H_2O or KCl.
5. Place tubes on platform shaker for 10 minutes at 150 opm.
6. Centrifuge at 3000 rpm for 2 minutes then remove and discard upper phase by suction.
7. Wash the lower phase with the addition of 0.5 mL of B&D synthetic upper phase and vortexing briefly.
8. Centrifuge at 3000 rpm for 2 minutes then remove and discard upper phase by suction.
9. Dry samples (lower phase) under N_2 at 40°C (add water to heating block around tube to aid in evaporation). Periodically vortex the samples during the drying down process to ensure the highest recovery possible.
10. Resuspend extracts in 150 µL of MeOH containing 10 mM ammonium formate.

APPENDIX II: Procedure for glycolipid, phospholipid and ganglioside extraction from plasma (Folch extraction).

1. Add 100 µL plasma to a 12 mL glass tube with black screw cap lid.
2. Add 2 mL $CHCl_3$/MeOH (2:1) (at least 20 volumes of $CHCl_3$/MeOH to each sample).
3. Add internal standards to each sample 2 µL (d3) C16:0 LC (200 µM); 2µL (d3) C16:0 GC (200 µM), and 2 µL GM2 (200 µM), 6.25 µL CTH C17:0 (64 µM); 2 µL Cer C17:0 (200 µM); 2 µL PC (200 µM).
4. Shake for 10 minutes at 150 rpm. Stand on the bench at room temperature for at least 50 minutes.
5. Partition with the addition of 0.2 volumes (ie. 0.4 mL) of milliQ H_2O and vortex.
6. Centrifuge at 4000 x g for 10 minutes then gently remove upper aqueous layer, transferring it to a clean glass tube with a glass pipette for use in the ganglioside extraction and set aside (*refer to ganglioside extraction*). Carefully remove and discard the protein interphase.
7. Dry samples (lower phase) under N_2 at 40°C.
8. Resuspend samples in 20 µL methanol and add 0.18 mL $CHCl_3$ (containing 1% ethanol) and vortex to ensure sample is resuspended.
9. Pre-wash silica reverse phase columns (100 mg) with 3 mL acetone/methanol (9:1) followed by 3 mL $CHCl_3$ (containing 1% ethanol).
10. Load sample with a glass pipette and allow it to completely enter the solid phase, then wash with 3 mL $CHCl_3$ (containing 1% ethanol) (neutral lipids (ceramide) will go through and glycolipids/phospholipids will bind to the column).
11. Elute the glycolipids and phospholipids from the column into a clean 12 mL glass tube with black screw cap lid with 3 mL acetone/methanol (9:1) and vacuum dry columns briefly. (LC and GC internal standards are present in this fraction.)

12. Elute the phospholipids from the column into clean 12 mL glass tube with black screw cap lid with 3 mL methanol and vacuum dry columns briefly. (PC internal standard is present in this fraction if used.)

Note: Omitting step 10 will result in the glycolipids and phospholipids being eluted together.

13. Dry samples under N_2 at 40°C
14. Resuspend samples in 100 µL MeOH and store at –20°C.
15. Prior to running on the mass spectrometer resuspend samples into a final volume of 200 µL methanol containing 10 mM ammonium formate.

Ganglioside extraction

1. Follow glycolipid and phospholipid extraction procedure to step 6, taking upper aqueous phase from Folch extraction following H_2O partition.
2. Prime 25 mg C18 columns with 3 x 1 mL MeOH, followed by 3x1 mL MQ water.
3. Load upper phase to column with a glass pipette and allow solution to completely enter the solid phase of the column, then wash with 3 x 1 mL MQ water.
4. Elute gangliosides from the column into a clean 12 mL glass tube with black screw cap lid with 2 x 1 mL MeOH and vacuum dry columns briefly.
5. Dry samples under N_2 at 40°C
6. Store samples at –20°C.
7. Prior to running on the mass spectrometer resuspend in 200 µL methanol containing 10 mM ammonium formate.

APPENDIX III: Procedure for Extraction of Glycosphingolipids from Guthrie Spots

Materials and Reagents:

Isopropanol standards mixture:

1.0 µM Phosphatidylcholine C14:0/C14:0 (MW=678)

1.0 µM Glucosylceramide(d3) C18:0 (MW=703.8)

1.0 µM Lactosylceramide(d3) C16:0 (MW=865.6)

1.0 µM Ceramide C17:0 (MW=252.7)

1.0 µM Tri-hexose ceramide CTH C17:0 (MW=1038.9)

1.0 µM Monosialoganglioside GM2 (MW=1384.9)

1 x 1 mL 96 deep-well, v-bottom tray (polypropylene) and lid

1 x 250 µL v-bottom tray

Multichannel pipette

Plate-shaker

Experimental Procedure:

1. Place two 3 mm blood spots per sample in each well of a 96 deep-well, v-bottom tray.
2. Add 200 µL isopropanol containing standards (200 pmol of each standard) to each sample.
3. Cover tray with polypropylene plastic lid and shake samples for 2 hours on amplitude setting 09 and form setting 99.
4. Remove 200 µL from samples into a 1 x 250 µL v-bottom tray leaving blood spots behind.
5. Dry down samples over N_2.
6. Resuspend extracts in 100 µL of MeOH containing 10 mM ammonium formate.
7. Cover plate with alfoil and analyse samples by mass spectrometry.

EXAMPLE 3 MONITORING OF THERAPY FOR GAUCHER DISEASE USING SPHINGOLIPID AND PHOSPHOLIPID ANALYSIS

This report provides a detailed analysis of the initial trial of our developed methodology to monitor enzyme replacement therapy (ERT) in Gaucher disease using dried blood spots.

Patient samples: Dried blood spots were collected from Gaucher patients receiving ERT for up to 10 years. In addition, dried blood spots have been collected from patients not receiving ERT. Control samples were collected from healthy individuals. Total sample numbers are as shown in Table 10.

Sample preparation: From each Guthrie card sample 2x3 mm dried blood spots were punched and the lipids were eluted (16h) with 200 µL of isopropanol containing 200 nmol of each internal standard; Cer C17:0, GC(*d3*)C16:0, LC(*d3*)C16:0, PC C14:0, PG C14:0/14:0. The blood spots were removed and the isopropanol dried under a stream of nitrogen. Lipids were redissolved in 100 µL of methanol containing 10 mM NH_4COOH for analysis by mass spectrometry.

Mass spectrometry: Mass spectrometric analysis of lipids was performed using a PE Sciex API 3000 triple-quadrupole mass spectrometer with a turbo-ionspray source and Analyst data system (PE Sciex, Concord, Ontario, Canada). Samples (20 µL) were injected into the electrospray source with a Gilson 233 autosampler using a carrying solvent of methanol at a flow rate of 80 µL/minute. For all analytes nitrogen was used as the collision gas at a pressure 2×10^{-5} Torr. Lipids were analysed in +ve ion mode for sphingolipids and phosphatidylcholine and –ve ion mode for all other phospholipids. Determination of lipids was performed using the multiple-reaction monitoring (MRM) mode. Seventeen different glycosphingolipid and ceramide species in addition to 36 phospholipid species were monitored using the ion pairs shown in Table 11 and 12. Each ion pair was monitored for 100 milliseconds and the measurements were repeated and averaged over the injection period. Determination of lipids was achieved by relating the peak height of each lipid ion

signal to the peak height of the signal from the corresponding internal standard (Table 11 and 12).

Results: To determine which analytes were potentially useful markers for monitoring Gaucher disease, the patients were grouped into control (group 1, n=22), Gaucher patients receiving ERT (group 2, n=68), and untreated Gaucher patients (group 3, n=20). Mann-Whitney U values were then calculated for each analyte to determine the difference between the control and untreated patients, control and treated patients, and treated and untreated patients. These results are shown in Table 13.

We observed that, in addition to the expected elevation of glucosylceramide (GC) in the untreated Gaucher patients compared to controls, there were significant differences in the level of ceramide C16:0, CTH C24:0 and the sphingomyelin species C16:0, C22:0 and C24:0 (all significant to the 0.01 level). With the exception of the ceramide C16:0, the same markers also showed a significant difference between treated and untreated Gaucher patients. Of the lactosylceramide species only the C16:0 and C22:0-OH species showed a significant difference between control and untreated patients (significant to the 0.05 level) (Table 13). While the GC and ceramide species were elevated in the Gaucher patient group compared to the control group, the LC, CTH and SM species showed a decrease in the Gaucher group. Many of the phospholipid species showed a significant difference between the control and Gaucher groups All of the phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine species and many of the phosphatidylglycerol and phosphatidylinositol species were significantly decreased in the Gaucher patient group compared to the control group (Table 13). Many of these analytes were also decreased in the treated Gaucher patient group. For those analytes where a significant difference was observed between the control and Gaucher groups, the levels in the treated patients generally fell between the control and untreated patients. In each case ERT has partially normalised the lipid levels, although not in all patients.

Although the observed differences between control and untreated patients are significant there is still considerable overlap between the two populations. This is due, at least in part, to the range of lipid levels in the control and patient groups. To improve the discrimination of the markers we investigated the use of multiple markers by calculating Mann-Whitney U values for a number of ratios of different lipid species (Table 14).

In all ratios the Mann-Whitney U values were decreased compared to the GC C16:0 values or other single analytes (compare Table 14 with Table 13). Clearly, the use of multiple analytes or lipid profiles provides a better representation of lipid metabolism in control and Gaucher patients.

Discussion: In this study we have provided evidence that the primary storage substrate GC is a useful marker for monitoring Gaucher disease. We observe an increased level of GC in dried blood spots from untreated patients compared to controls and a normalisation of GC levels after ERT. This is an expected outcome, based on the known biochemistry of Gaucher disease. Somewhat less expected is the elevation in ceramide and the decrease in LC and sphingomyelin. We have previously reported that LC is decreased in the plasma of Gaucher patients and that the ratio of GC/LC provides a better discrimination of Gaucher patients from controls than the GC levels on their own (Whitfield et al 2002). In these preliminary studies we have identified that other lipids are also affected, these include not only ceramide and sphingomyelin but also a number of phospholipids. We have also shown that using a combination of these analytes with the GC and LC levels, provides greater discrimination and potentially a better mechanism for monitoring ERT in Gaucher disease than the use of individual analytes.

Table 10. Patient and control samples included in this trial

Patient group	Number
Control	19
Treated Gaucher	68
Untreated Gaucher	20

Table 11. Lipid analytes used for Gaucher Monitoring

Lipid analytes[a]	Internal standard	MRM ion pairs (*m/z*)
Cer C16:0	Cer C17:0	538.7/264.4
Cer C24:0	Cer C17:0	650.7/264.4
Cer C24:1	Cer C17:0	648.7/264.4
Cer C17:0 (internal standard)		552.7/264.4
GC C16:0	GC(*d3*)C16:0	700.6/264.4
GC C22:0	GC(*d3*)C16:0	784.7/264.4
GC C24:0	GC(*d3*)C16:0	812.7/264.4
GC C24:1	GC(*d3*)C16:0	810.8/264.4
GC(*d3*)C16:0 (internal standard)		703.8/264.4
LC C16:0	LC(*d3*)C16:0	862.4/264.4
LC C24:0	LC(*d3*)C16:0	974.8/264.4
LC C24:1	LC(*d3*)C16:0	972.8/264.4
CTH C16:0	LC(*d3*)C16:0	1024.1/264.4
CTH C22:0	LC(*d3*)C16:0	1108.1/264.4
CTH C24:0	LC(*d3*)C16:0	1136.6/264.4
CTH C24:1	LC(*d3*)C16:0	1134.1/264.4
LC(*d3*)C16:0 (internal standard)		865.6/264.4
SM C16:0	PC C14:0	703.9/184.1
SM C22:0	PC C14:0	787.8/184.1
SM C24:0	PC C14:0	815.8/184.1
PC C14:0 (internal standard)		678.5/184.1

[a] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihoxoside, SM = sphingomyelin, PC = phosphatidylcholine

Table 12. Phospholipid analytes used for Gaucher Monitoring

Lipid analytes[a]	Internal standard	MRM ion pairs (*m/z*)
PC C32:0	PC C14:0	734.7/184
PC C32:1	PC C14:0	732.7/184
PC C34:1	PC C14:0	760.6/184
PC C34:2	PC C14:0	758.5/184
PC C36:2	PC C14:0	786.6/184
PC C36:4	PC C14:0	782.6/184
PC C38:4	PC C14:0	810.8/184
PC C14:0 (internal standard)		678.5/184
PE C18:0/20:4	PG C14:0/14:0	766.6/303.4
PE C18:1/18:1	PG C14:0/14:0	742.6/281.1
PG C16:0/18:1	PG C14:0/14:0	747.6/255.8
PG C16:0/22:6	PG C14:0/14:0	793.5/255.5
PG C16:1/18:1	PG C14:0/14:0	745.5/281.5
PG C16:1/20:4	PG C14:0/14:0	767.4/253.5
PG C18:1/18:0	PG C14:0/14:0	775.6/281.0
PG C18:1/18:1	PG C14:0/14:0	773.4/281.0
PG C18:1/18:2	PG C14:0/14:0	771.8/281.2
PG C18:1/20:4	PG C14:0/14:0	795.6/303.5
PG C18:1/22:5	PG C14:0/14:0	821.8/281.0
PG C18:1/22:6	PG C14:0/14:0	819.7/281.0
PG C18:2/22:6	PG C14:0/14:0	817.6/279.0
PG C20:4/22:6	PG C14:0/14:0	841.5/303.5
PG C22:6/22:5	PG C14:0/14:0	867.5/329.3
PG C22:6/22:6	PG C14:0/14:0	865.6/327.1
PI C16:0/18:0	PG C14:0/14:0	835.4/283.2
PI C16:0/20:4	PG C14:0/14:0	857.6/255.2
PI C18:0/18:0	PG C14:0/14:0	865.6/283.3
PI C18:0/18:1	PG C14:0/14:0	863.6/283.1
PI C18:0/20:4	PG C14:0/14:0	885.6/283.1
PI C18:0/22:4	PG C14:0/14:0	913.7/283.6
PI C18:0/22:5	PG C14:0/14:0	911.6/283.3
PI C18:1/18:1	PG C14:0/14:0	861.4/281.1
PI C18:1/20:4	PG C14:0/14:0	883.6/281.2
PS C16:0/16:0	PG C14:0/14:0	734.3/255.5
PS C18:0/20:4	PG C14:0/14:0	810.6/283.3
PS C18:1/18:0	PG C14:0/14:0	788.4/283.1
PG C14:0/14:0 (internal standard)		591.5/227.4

[a] PC = phosphatidylcholine, PG = phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine, PE phosphatidylethanolamine

5

Table 13. Mann-Whitney U values for lipid analytes between controls[a], untreated Gaucher patients[b] and Gaucher patients treated with enzyme replacement therapy[c].

Analyte[d]	Control vs Gaucher		Control vs Gaucher treated		Gaucher vs Gaucher Treated	
	M-W U[e]	Sig.[f]	M-W U	Sig.	M-W U	Sig.
Cer C16:0	37	0.000	294	0.000	584	0.339
Cer C24:0	114	0.033	481	0.090	597	0.409
Cer C24:1	153	0.299	589	0.558	627	0.598
GC C16:0	25	0.000	310	0.001	332	0.001
GC C22:0	112	0.028	580	0.498	488	0.056
GC C24:0	125	0.068	606	0.681	493	0.063
GC C24:1	71	0.001	334	0.001	391	0.004
LC C16:0	120	0.049	556	0.355	534	0.146
LC C20:0	163	0.448	595	0.600	544	0.176
LC C22:0	178	0.736	589	0.558	667	0.897
LC C22:0-OH	111	0.026	578	0.485	494	0.064
LC C24:0	187	0.933	625	0.829	665	0.881
LC C24:1	166	0.500	596	0.607	670	0.921
(LC) CTH C16:0	124	0.064	594	0.593	508	0.087
(LC) CTH C18:0	174	0.653	563	0.394	622	0.564
(LC) CTH C20:0	139	0.152	440	0.034	611	0.492
(LC) CTH C22:0	115	0.035	573	0.453	486	0.053
(LC) CTH C24:0	76	0.001	462	0.059	418	0.009
(LC) CTH C24:1 (1134.9/264.4)	131	0.097	581	0.504	390	0.004
SM C16:0	69	0.001	497	0.126	379	0.003
SM C22:0	68	0.001	479	0.086	397	0.005
SM C24:0	85	0.003	353	0.003	464	0.031
PC C32:0	161	0.415	521	0.199	475	0.041
PC C32:1	47	0.000	236	0.000	678	0.984
PC C34:1	82	0.002	338	0.002	553	0.206
PC C34:2	70	0.001	432	0.028	437	0.016
PC C36:2	69	0.001	503	0.142	384	0.003
PC C36:4	48	0.000	322	0.001	401	0.005
PC C38:4	56	0.000	431	0.027	362	0.002
PE 18:0/20:4 (766.6/303.4)	57	0.000	509	0.025	325	0.000
PE 18:1/18:1 (742.6/281.1)	97	0.002	430	0.003	475.5	0.042
PG 16:0/18:1 (747.6/255.8)	160	0.131	715	0.757	538	0.157
PG 16:0/22:6 (793.5/255.5)	136.5	0.035	701	0.659	480	0.046
PG 16:1/18:1 (745.5/281.5)	97	0.002	386	0.001	541	0.166
PG 16:1/20:4 (767.4/253.5)	127	0.019	319	0.000	562	0.240
PG 18:1/18:0 (775.6/281.0)	133	0.028	604	0.176	539	0.160
PG 18:1/18:1 (773.4/281.0)	199	0.597	649	0.353	527	0.128

PG 18:1/18:2 (771.8/281.2)	104	0.003	488	0.015	520	0.111
PG 18:1/20:4 (795.6/303.5)	104	0.003	739	0.933	349	0.001
PG 18:1/22.:5 (821.8/281.0)	146	0.062	598	0.159	578	0.310
PG 18:1/22:6 (819.7/281.0)	140	0.044	540	0.051	600	0.426
PG 18:2/22:6 (817.6/279.0)	99	0.002	601	0.168	419	0.009
PG 20:4/22:6 (841.5/303.5)	82	0.001	692	0.599	316	0.000
PG 22:6/22:5 (867.5/329.3)	168	0.190	669.5	0.461	555	0.213
PG 22:6/22:6 (865.6/327.1)	174	0.247	491	0.016	605	0.455
PI 16:0/18:0 (835.4/283.2)	107	0.004	515	0.029	483	0.050
PI.16:0/20:4 (857.6/255.2)	96	0.002	532	0.043	501	0.075
PI 18:0/18:0 (865.6/283.3	125	0.017	463	0.007	617	0.530
PI 18:0/18:1 (863.6/283.1	69	0.000	359	0.000	607	0.467
PI 18:0/20:4 (885.6/283.1)	114	0.008	438	0.004	559	0.228
PI 18:0/22:4 (913.7/283.6)	166	0.174	488	0.015	671	0.929
PI 18:0/22:5 (911.6/283.3)	78	0.000	215	0.000	620	0.550
PI 18:1/18:1 (861.4/281.1)	99	0.002	499	0.019	522	0.116
PI 18:1/20:4 (883.6/281.2)	132	0.027	557	0.073	566	0.256
PS 16:0/16:0 (734.3/255.5)	188	0.420	589	0.135	605	0.455
PS 18:0/20:4 (810.6/283.3)	85	0.001	417	0.002	444	0.019
PS 18:1/18:0 (788.4/283.1)	81	0.000	409	0.001	556	0.217
Total Cer	150	0.261	597	0.615	632	0.633
Total GC	49	0.000	330	0.001	362	0.002
Total LC	170	0.574	619	0.781	630	0.619
Total CTH	103	0.015	519	0.192	475	0.041
Total SM	68	0.001	443	0.037	399	0.005
Total PC	75	0.001	397	0.011	445	0.019

[a] controls n=22
[b] untreated n= 20
[c] treated n= 68
[d] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihoxoside, SM = sphingomyelin, PC = phosphatidylcholine, PG = phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine, PE phosphatidylethanolamine
[e] Mann-Whitney U values
[f] significance (two-tailed)

Table 14. Mann-Whitney U values for lipid analyte ratios between controls[a], untreated Gaucher patients[b] and Gaucher patients treated with enzyme replacement therapy[c].

Analyte Ratio	Control vs Gaucher		Control vs Treated		Treated vs untreated	
	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.
GC C16:0 / PE 18:0/20:4	28	0.000	241	0.000	291	0.000
GC C16:0 / PG 18:1/18:2	25	0.000	260	0.000	322	0.000
GC C16:0 / PG 20:4/20:6	19	0.000	344	0.002	229	0.000
GC C16:0 / PI 18:0/18:1	20	0.000	184	0.000	373	0.002
(Cer C16:0*GC C16:0) / (CTH C24:0*SM C16:0)	17	0.000	157	0.000	259	0.000
(Cer C16:0*GC C16:0) / (CTH C24:0*SM C16:0*PC32:1*PG20:4/22:6* PI18:0/18:1)	23	0.000	205	0.000	307	0.000
(Cer C16:0*GC C16:0) / (PC 32:1*PG 20:4/22:6*PI 18:0/18:1)	12	0.000	159	0.000	366	0.002

[a] controls n=22
[b] untreated n= 20
[c] treated n= 68
[d] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihoxoside, SM = sphingomyelin, PC = phosphatidylcholine, PG = phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine, PE phosphatidylethanolamine
[e] Mann-Whitney U values
[f] significance (two-tailed)

EXAMPLE 4

DIAGNOSIS OF FABRY DISEASE USING SPHINGOLIPID AND PHOSPHOLIPID ANALYSIS

This report summarises the results of analyses performed on urine, from controls, Fabry and Fabry heterozygotes, including analysis of phospholipids.

MATERIALS AND METHODS

Patient samples: Urine samples have been collected from 14 Fabry patients (two of whom have had renal transplants), 14 Fabry heterozygotes (three of whom had reported clinical symptoms) and 29 unaffected controls.

Sample preparation and analysis: Urine samples were prepared as described

To 1.5 mL urine add 5.6 mL $CHCl_3$/MeOH (1:2)

Add 400 pmol internal standards to each sample; 2 µL (d3) C16:0 LC (200 µM); 2 µL (d3) C16:0 GC (200 µM), 2 µL Cer C17:0 (200 µM), 2 µL PC (200 µM), 2 µL PG (200 µM) and 2 µL PI (200 µM).

Place tubes on platform shaker for 10 minutes at 150 opm. Stand tubes at room temperature for at least 50 minutes.

Partition with the addition of 1.9 mL $CHCl_3$ and 1.9 mL milliQ H_2O or KCl.

Place tubes on platform shaker for 10 minutes at 150 opm.

Centrifuge at 3000 rpm for 2 minutes then remove and discard upper phase by suction.

Wash the lower phase with the addition of 0.5 mL of Bligh-Dyer synthetic upper phase and vortexing briefly.

Centrifuge at 3000 rpm for 2 minutes then remove and discard upper phase by suction.

Dry samples (lower phase) under N_2 at 40^0C (add water to heating block around tube to aid in evaporation). Periodically vortex the samples during the drying down process to ensure the highest recovery possible.

Resuspend extracts in 150 µL of MeOH containing 10 mM ammonium formate.

Mass spectrometry: Mass spectrometric analysis of lipids was performed using a PE Sciex API 3000 triple-quadrupole mass spectrometer with a turbo-ionspray source and Analyst data system (PE Sciex, Concord, Ontario, Canada). Samples (20 µL) were injected into the electrospray source with a Gilson 233 autosampler using a carrying solvent of methanol at a flow rate of 80 µL/minute. For all analytes nitrogen was used as the collision gas at a pressure 2 x 10^{-5} Torr. Lipids were analysed in +ve ion mode for sphingolipids and phosphatidylcholine and –ve ion mode for all other phospholipids. Determination of lipids was performed using the multiple-reaction monitoring (MRM) mode. Seventeen different glycosphingolipid and ceramide species in addition to 36 phospholipid species were monitored using the ion pairs shown in Table 15 and 16. Each ion pair was monitored for 100 milliseconds and the measurements were repeated and averaged over the injection period. Determination of lipids was achieved by relating the peak height of each lipid ion signal to the peak height of the signal from the corresponding internal standard

RESULTS

Analysis of Urine: Lipid profiling of the urine samples from control, Fabry and Fabry heterozygotes (Fabry het) has been performed. In all, 52 lipid species were determined including ceramide (Cer), glucosylceramide (GC), lactosylceramide (LC), trihexosylceramide (CTH), sphingomyelin (SM) and phosphatidylcholine (PC), phosphatidylglygerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and phosphatidylserine (PS) species. Appropriate internal standards were used that provide quantification of these species (expressed as nmol/L urine). PC was included as a general marker of urinary sediment and all lipid species were subsequently corrected for total PC content and expressed as nmol/umol PC.

Table 17 shows the Mann-Whitney U values for each of the two patient groups compared to the control group and of the patient groups compared to each other. The data shows multiple analytes to be significantly different between the control and patient groups. Primarily LC CTH, PC and PG species show major differences between control and Fabry groups. Fewer

species show significant differences between control and Fabry Het groups but still 11 lipid species show a significance less than 0.01.

Table 18 shows the Mann-Whitney U values for different lipid ratios involving 2 or more lipid species. In most instances the ratios provide better discrimination than the individual analytes involved (based on the Mann-Whitney U values.

DISCUSSION

In this study we have provided evidence that the primary storage substrate CTH is a useful marker for diagnosis of Fabry disease. We observe an increased level of CTH in urine from most Fabry patients. This is an expected outcome, based on the known biochemistry of Fabry disease. Somewhat less expected is the elevation in all of the PC and PG species as well as two ceramide species and two of the three sphingomyelin species. In these preliminary studies we have identified that in addition to CTH, other lipids are also affected, these include not only ceramide and sphingomyelin but also a number of phospholipids. We have also shown that using a combination of these analytes either alone or with the CTH levels, provides greater discrimination and potentially a better mechanism for diagnosis of Fabry and identification of Fabry heterozygotes than the use of individual analytes.

Table 15. Lipid analytes used for Fabry urine analysis

Lipid analytes[a]	Internal standard	MRM ion pairs (*m/z*)
Cer C16:0	Cer C17:0	538.7/264.4
Cer C24:0	Cer C17:0	650.7/264.4
Cer C24:1	Cer C17:0	648.7/264.4
Cer C17:0 (internal standard)		552.7/264.4
GC C16:0	GC(*d3*)C16:0	700.6/264.4
GC C22:0	GC(*d3*)C16:0	784.7/264.4
GC C24:0	GC(*d3*)C16:0	812.7/264.4
GC C24:1	GC(*d3*)C16:0	810.8/264.4
GC(*d3*)C16:0 (internal standard)		703.8/264.4
LC C16:0	LC(*d3*)C16:0	862.4/264.4
LC C24:0	LC(*d3*)C16:0	974.8/264.4
LC C24:1	LC(*d3*)C16:0	972.8/264.4
CTH C16:0	LC(*d3*)C16:0	1024.1/264.4
CTH C22:0	LC(*d3*)C16:0	1108.1/264.4
CTH C24:0	LC(*d3*)C16:0	1136.6/264.4
CTH C24:1	LC(*d3*)C16:0	1134.1/264.4
LC(*d3*)C16:0 (internal standard)		865.6/264.4
SM C16:0	PC C14:0	703.9/184.1
SM C22:0	PC C14:0	787.8/184.1
SM C24:0	PC C14:0	815.8/184.1
PC C14:0 (internal standard)		678.5/184.1

[a] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihoxoside, SM = sphingomyelin, PC = phosphatidylcholine

5

Table 16. Phospholipid analytes used for Fabry urine analysis.

Lipid analytes[a]	Internal standard	MRM ion pairs (*m/z*)
PC C32:0	PC C14:0	734.7/184
PC C32:1	PC C14:0	732.7/184
PC C34:1	PC C14:0	760.6/184
PC C34:2	PC C14:0	758.5/184
PC C36:2	PC C14:0	786.6/184
PC C36:4	PC C14:0	782.6/184
PC C38:4	PC C14:0	810.8/184
PC C14:0 (internal standard)		678.5/184.1
PE C18:0/20:4	PG C14:0/14:0	766.6/303.4
PE C18:1/18:1	PG C14:0/14:0	742.6/281.1
PG C16:0/18:1	PG C14:0/14:0	747.6/255.8
PG C16:0/22:6	PG C14:0/14:0	793.5/255.5
PG C16:1/18:1	PG C14:0/14:0	745.5/281.5
PG C16:1/20:4	PG C14:0/14:0	767.4/253.5
PG C18:1/18:0	PG C14:0/14:0	775.6/281.0
PG C18:1/18:1	PG C14:0/14:0	773.4/281.0
PG C18:1/18:2	PG C14:0/14:0	771.8/281.2
PG C18:1/20:4	PG C14:0/14:0	795.6/303.5
PG C18:1/22:5	PG C14:0/14:0	821.8/281.0
PG C18:1/22:6	PG C14:0/14:0	819.7/281.0
PG C18:2/22:6	PG C14:0/14:0	817.6/279.0
PG C20:4/22:6	PG C14:0/14:0	841.5/303.5
PG C22:6/22:5	PG C14:0/14:0	867.5/329.3
PG C22:6/22:6	PG C14:0/14:0	865.6/327.1
PG C14:0/14:0 (internal standard)		591.5/227.4
PI C16:0/18:0	PI C16:0/16:0	835.4/283.2
PI C16:0/20:4	PI C16:0/16:0	857.6/255.2
PI C18:0/18:0	PI C16:0/16:0	865.6/283.3
PI C18:0/18:1	PI C16:0/16:0	863.6/283.1
PI C18:0/20:4	PI C16:0/16:0	885.6/283.1
PI C18:0/22:4	PI C16:0/16:0	913.7/283.6
PI C18:0/22:5	PI C16:0/16:0	911.6/283.3
PI C18:1/18:1	PI C16:0/16:0	861.4/281.1
PI C18:1/20:4	PI C16:0/16:0	883.6/281.2
PI C14:0/14:0 (internal standard)		751.5/227.4
PS C16:0/16:0	PG C14:0/14:0	734.3/255.5
PS C18:0/20:4	PG C14:0/14:0	810.6/283.3
PS C18:1/18:0	PG C14:0/14:0	788.4/283.1

[a] PC = phosphatidylcholine, PG = phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine, PE phosphatidylethanolamine

Table 17. Mann-Whitney U values for lipid analytes between controls[a], Fabry[b] and Fabry Hets[c].

Analyte[d]	Cont vs Fabry		Cont vs Het		Fabry vs Het	
	M-W U[e]	Sig[f]	M-W U	Sig	M-W U	Sig
Cer C16:0 (538.7/264.4)	119	0.029	189	0.717	62	0.098
Cer C24:0 (650.7/264.4)	132	0.066	175	0.468	52	0.035
Cer C24:1 (648.7/264.4)	70	0.001	187	0.678	37	0.005
Cer C20:0 (592.7/264.4)	155	0.213	168	0.364	59	0.073
Cer C20:1 (590.7/264.4)	193	0.795	124	0.041	46	0.017
Cer C23:0 (636.7/264.4)	144	0.126	143	0.120	53	0.039
Cer C23:1 (634.8/264.4)	160	0.265	146	0.140	51	0.031
GC C16:0 (700.6/264.4)	203	1.000	148	0.154	70	0.198
GC C22:0 (784.7/264.4)	152	0.186	89	0.003	48	0.022
GC C24:0 (812.7/264.4)	182	0.586	101	0.008	60	0.081
GC C24:1 (810.8/264.4)	137	0.087	143	0.120	41	0.009
LC C16:0 (862.4/264.4)	107	0.013	117	0.026	93	0.818
LC C20:0 (918.7/264.4)	66	0.000	196	0.856	29	0.002
LC C22:0 (946.7/264.4)	70	0.001	151	0.178	53	0.039
LC C22:0-OH (962.7/264.4)	75	0.001	166	0.338	44	0.013
LC C24:0 (974.8/264.4)	11	0.000	100	0.008	19	0.000
LC C24:1 (972.8/264.4)	41	0.000	98	0.007	66	0.141
(LC) CTH C16:0 (1024.8/264.4)	41	0.000	143	0.120	39	0.007
(LC) CTH C18:0 (1052.7/264.4)	18	0.000	157	0.233	20	0.000
(LC) CTH C20:0 (1080.9/264.4)	75	0.001	197	0.876	32	0.002
(LC) CTH C22:0 (1108.9/264.4)	47	0.000	96	0.006	48	0.022
(LC) CTH C24:0 (1136.9/264.4)	26	0.000	111	0.017	34	0.003
(LC) CTH C24:1 (1134.9/264.4)	43	0.000	106	0.012	46	0.017
PC C32:0 (734.7/184.1)	118	0.028	166	0.338	77	0.335
PC C32:1 (732.7/184.1)	58	0.000	167	0.351	55	0.048
PC C34:1 (760.6/184.1)	83	0.002	113	0.020	87	0.613
PC C34:2 (758.5/184.1)	86	0.002	183	0.604	34	0.003
PC C36:2 (786.6/184.1)	125	0.043	130	0.058	82	0.462
PC C36:4 (782.6/184.1)	87	0.003	202	0.979	59	0.073
PC C38:4 (810.8/184.1)	65	0.000	199	0.917	49	0.024
SM C16:0 (703.9/184.1)	182	0.586	160	0.265	84	0.520
SM C22:0 (787.8/184.1)	58	0.000	126	0.046	94	0.854
SM C24:0 (815.8/184.1)	44	0.000	100	0.008	97	0.963
PG C16:0/18:1 (747.6/255.8)	75	0.001	115	0.023	61	0.089
PG C16:0/22:6 (793.5/255.5)	70	0.001	154	0.204	54	0.043
PG C16:1/18:1 (745.5/281.5)	90	0.003	82	0.002	67	0.154
PG C16:1/20:4 (767.4/253.5)	137	0.087	193	0.795	70	0.198
PG C18:1/18:0 (775.6/281.0)	28	0.000	73	0.001	51	0.031
PG C18:1/18:1 (773.4/281.0)	15	0.000	73	0.001	38	0.006

Table 18. Mann-Whitney U values for lipid analyte ratios between controls[a], Fabry[b] and Fabry Hets[c].

Analyte[d]	Control v Fabry		Control v Fabry Het		Fabry v Fabry Het	
	M-W U[e]	Sig.[f]	M-W U	Sig.	M-W U	Sig.
CTH C24:1/SM C24:0	18	0.000	51	0.000	39	0.007
LC C24:1/GC C24:0	16	0.000	65	0.000	81	0.435
PC C38:4/PC C32:1	58	0.000	187	0.678	55	0.048
PC C36:4*PC C38:4/PC C32:1*PC C34:1	56	0.000	182	0.586	55	0.048
CTH C24:1/SM C24:0/LC C24:1/ GC C24:0	83	0.002	191	0.756	42	0.010
PG C18:1/18:1 /PS C18:1/18:0	2	0.000	35	0.000	14	0.000
PI C18:0/18:0 / PS C18:1/18:0	10	0.000	195	0.836	8	0.000
PG C18:1/18:1* PI C18:0/18:0 / PS C18:1/18:0	1	0.000	106	0.012	8	0.000
PG C18:1/18:1 / SM C18:1/18:0	4	0.000	16	0.000	33	0.003

[a] controls n=29
[b] Fabrt n= 14
[c] Fabry Het n= 14
[d] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihoxoside, SM = sphingomyelin, PC = phosphatidylcholine, PG = phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine, PB phosphatidylethanolamine
[e] Mann-Whitney U values
[f] significance (two-tailed)

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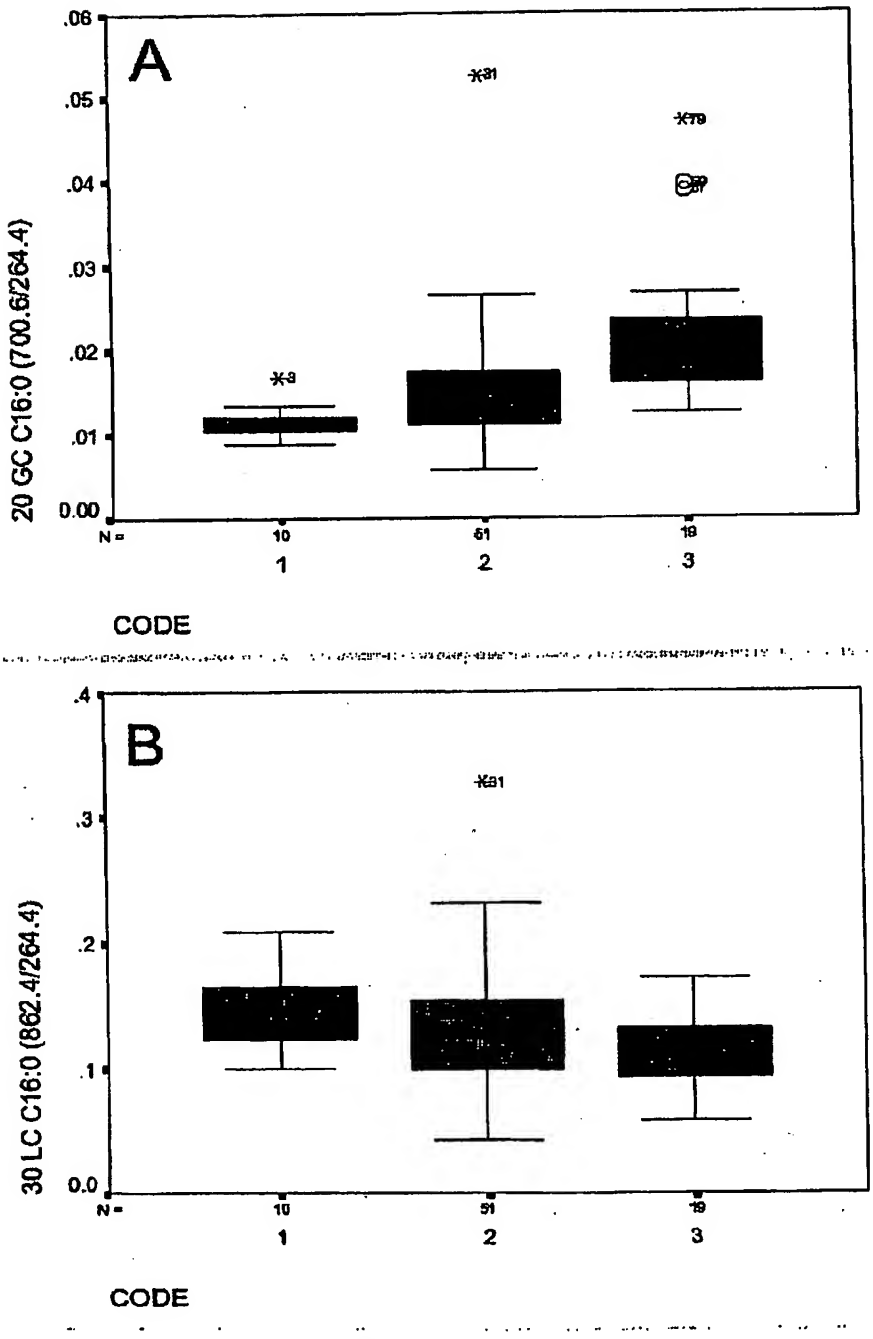
Various features of the invention have been particularly shown and described in connection with the exemplified embodiments of the invention, however, it must be understood that these particular arrangements merely illustrate and that the invention is not limited thereto and can include various modifications falling within the spirit and scope of the invention.

Dated this 31st day of March 2004

WOMEN'S AND CHILDREN'S HOSPITAL
By their Patent Attorneys
A.P.T. Patent and Trade Mark Attorneys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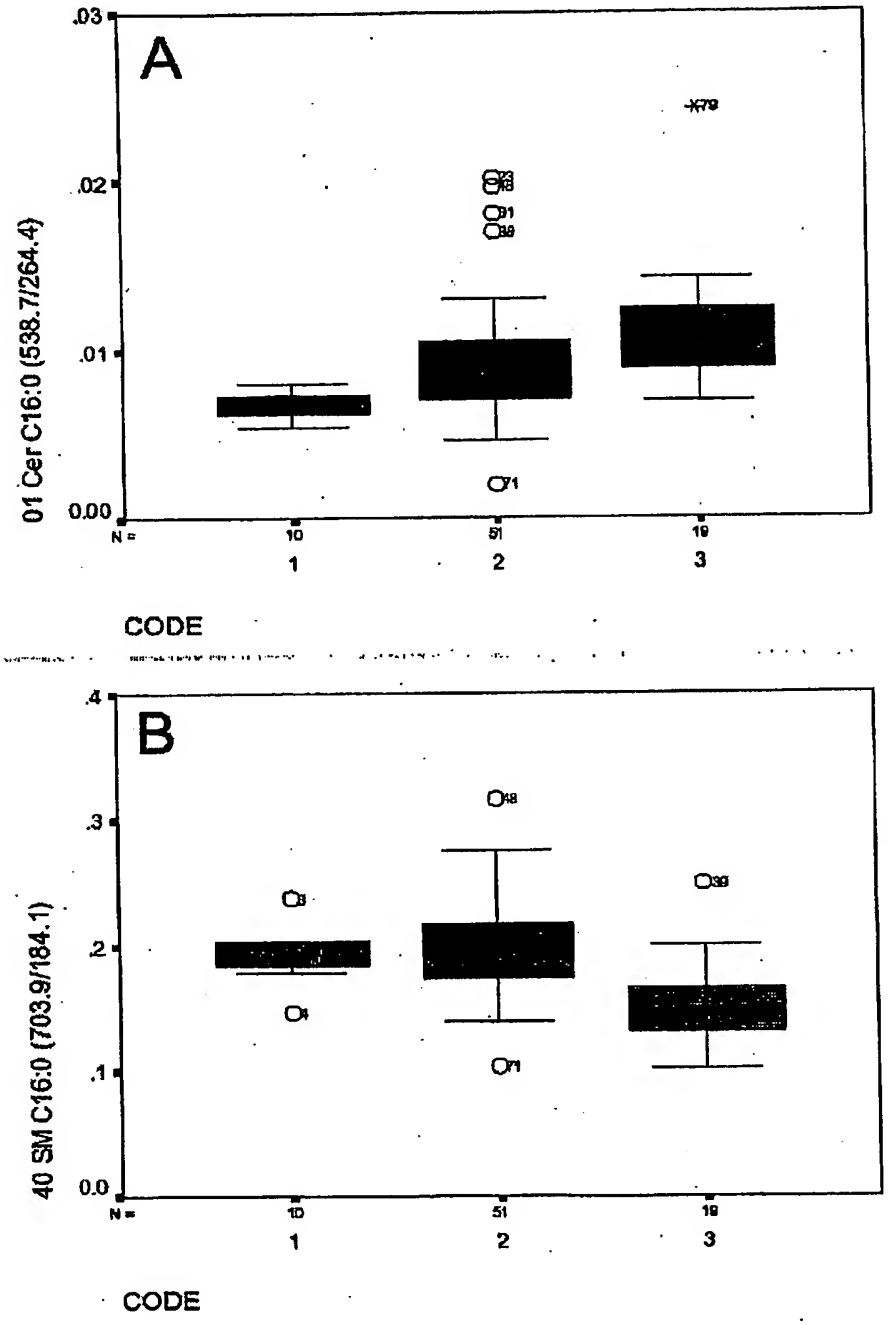
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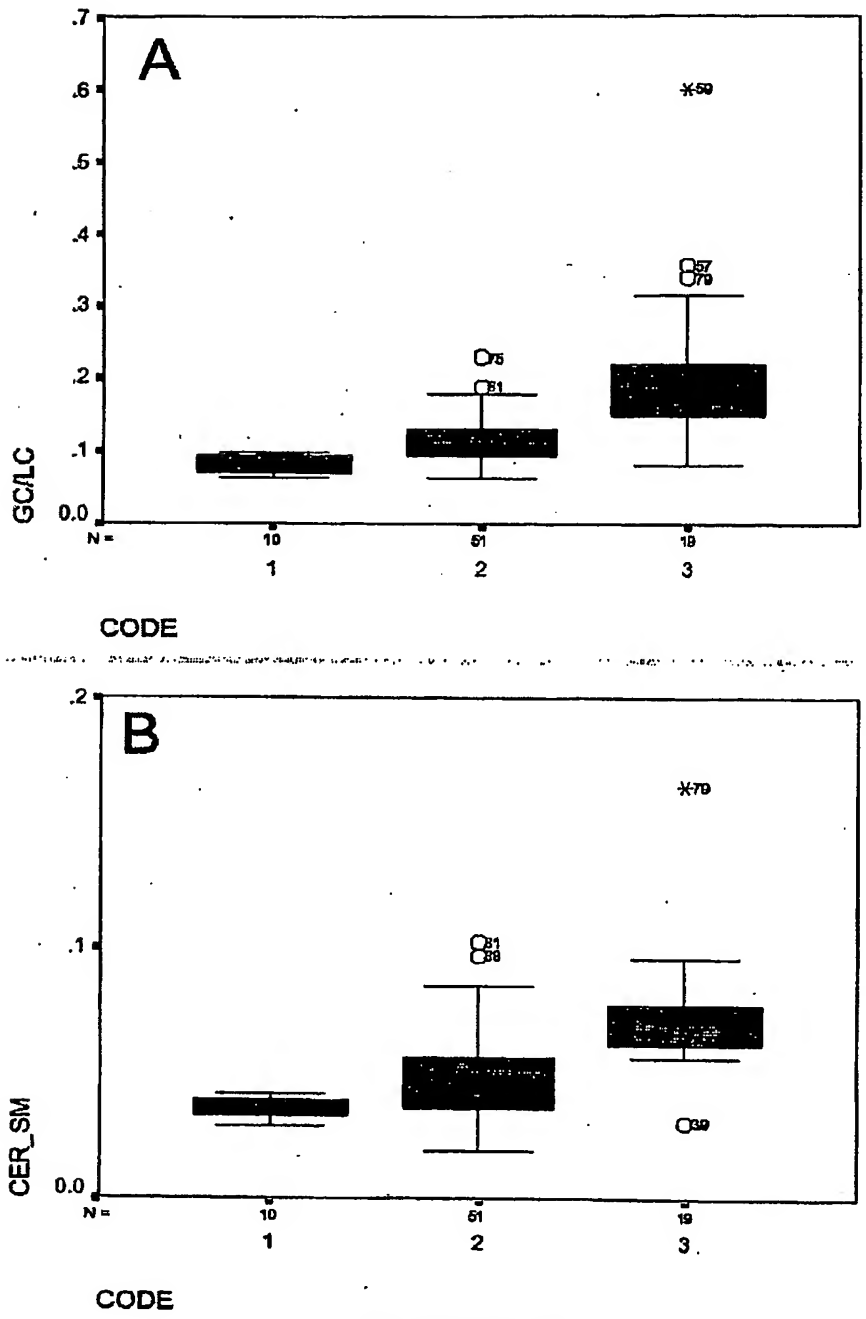
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FIGURE 3

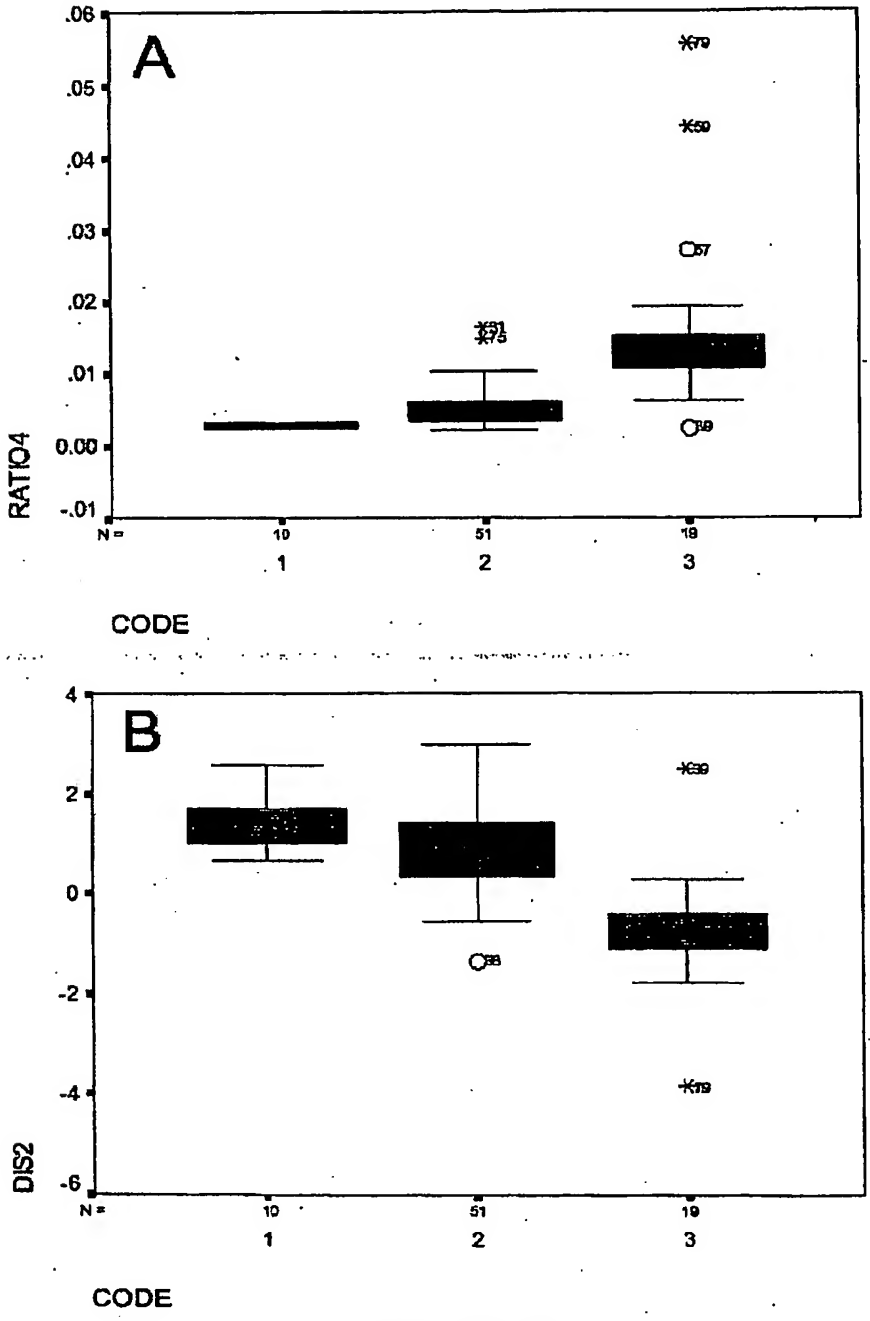
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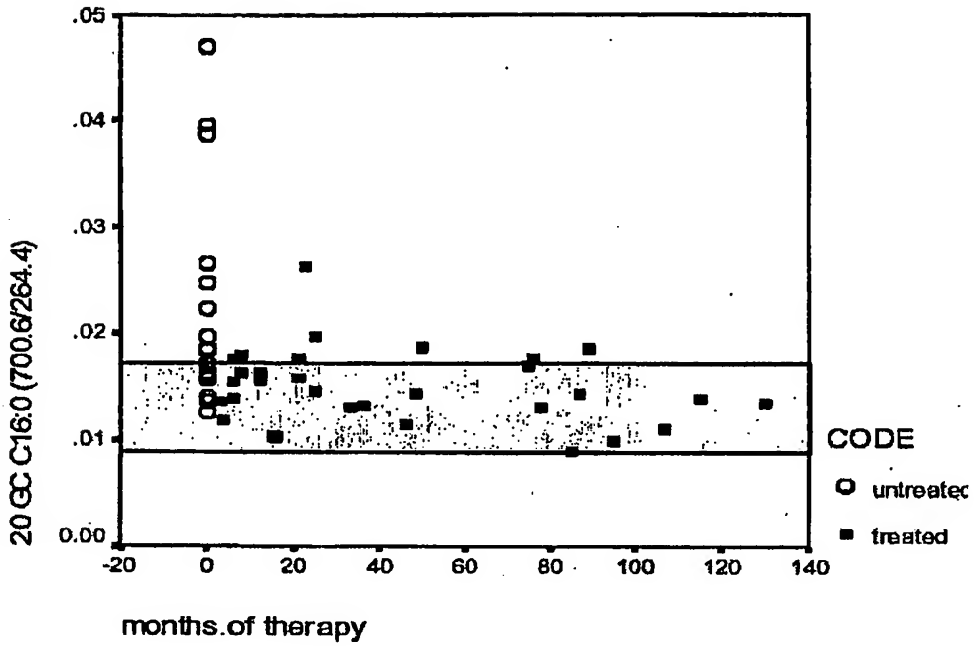
FIGURE 4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months.of therapy
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untreatec
treated

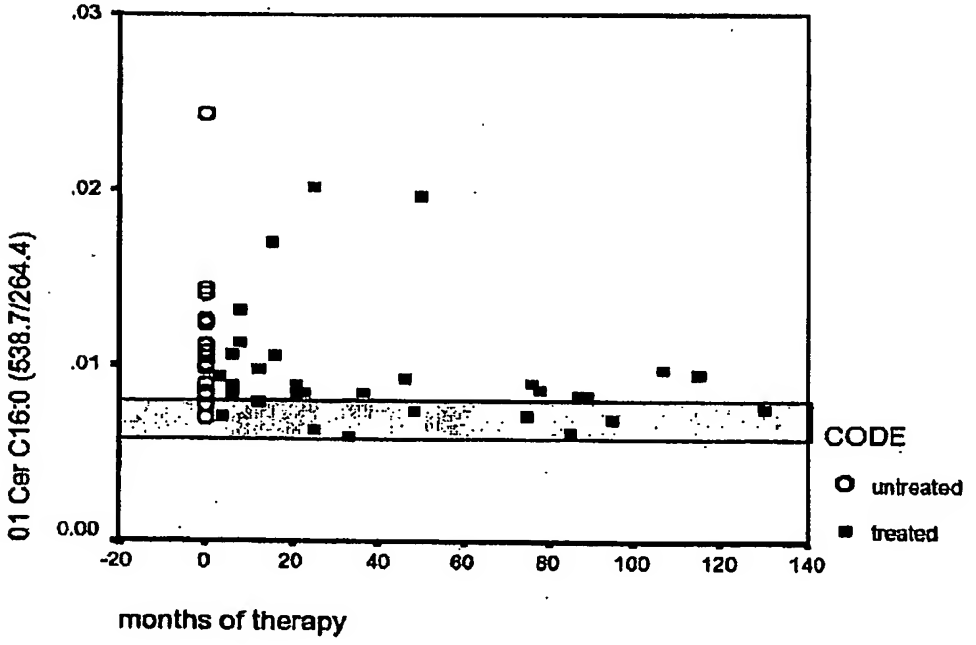

.03
.02
.01
0.00
01 Cer C16:0 (538.7/264.4)
-20
0
20
40
60
80
100
120
140
months of therapy
CODE
untreated
treated

FIGURE 5

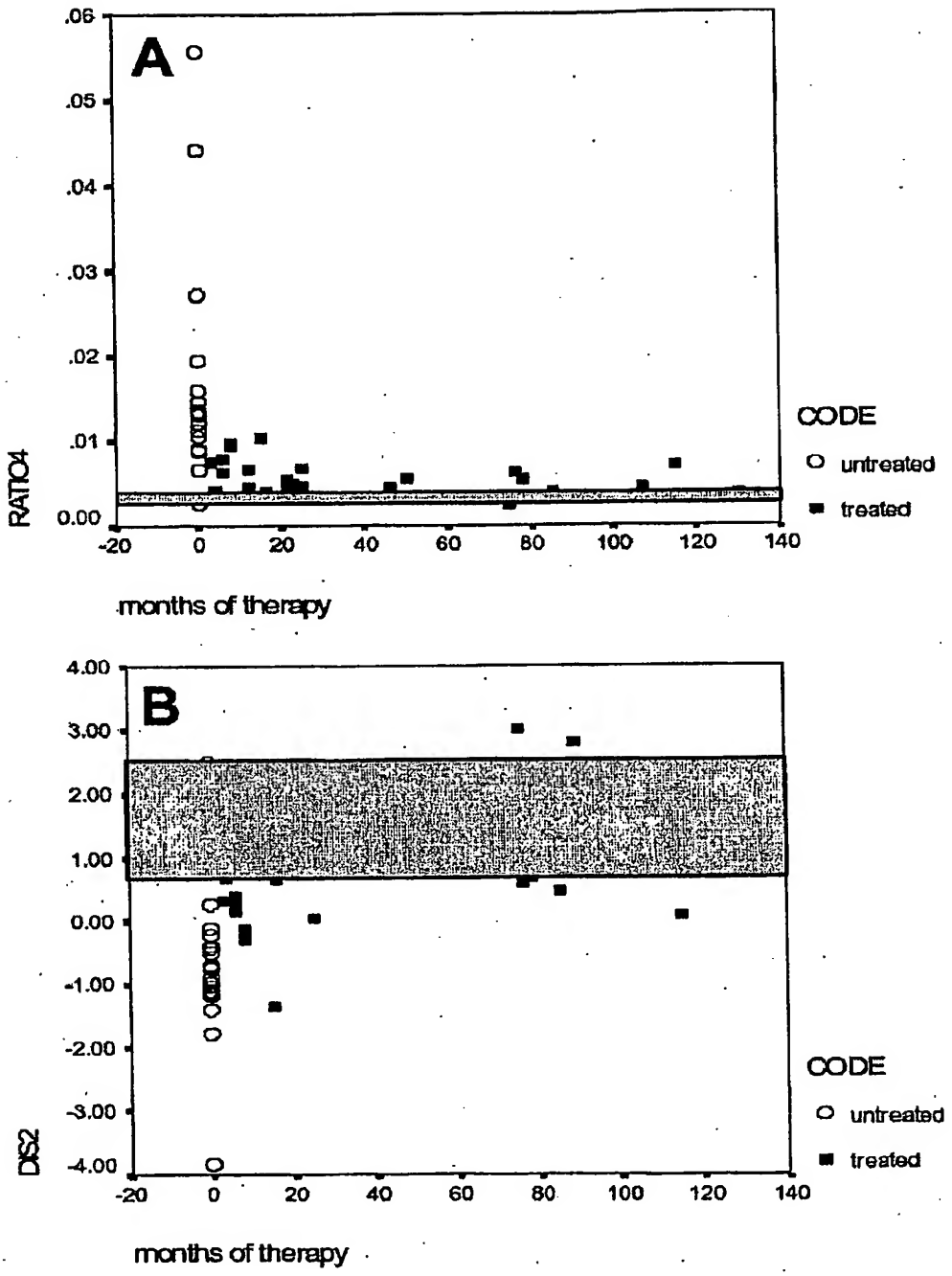

A
.06
.05
.04
.03
.02
.01
0.00
RATIO4
-20
0
20
40
60
80
100
120
140
months of therapy
CODE
untreated
treated
B
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
DIS2
-20
0
20
40
60
80
100
120
140
months of therapy
CODE
untreated
treated

FIGURE 6

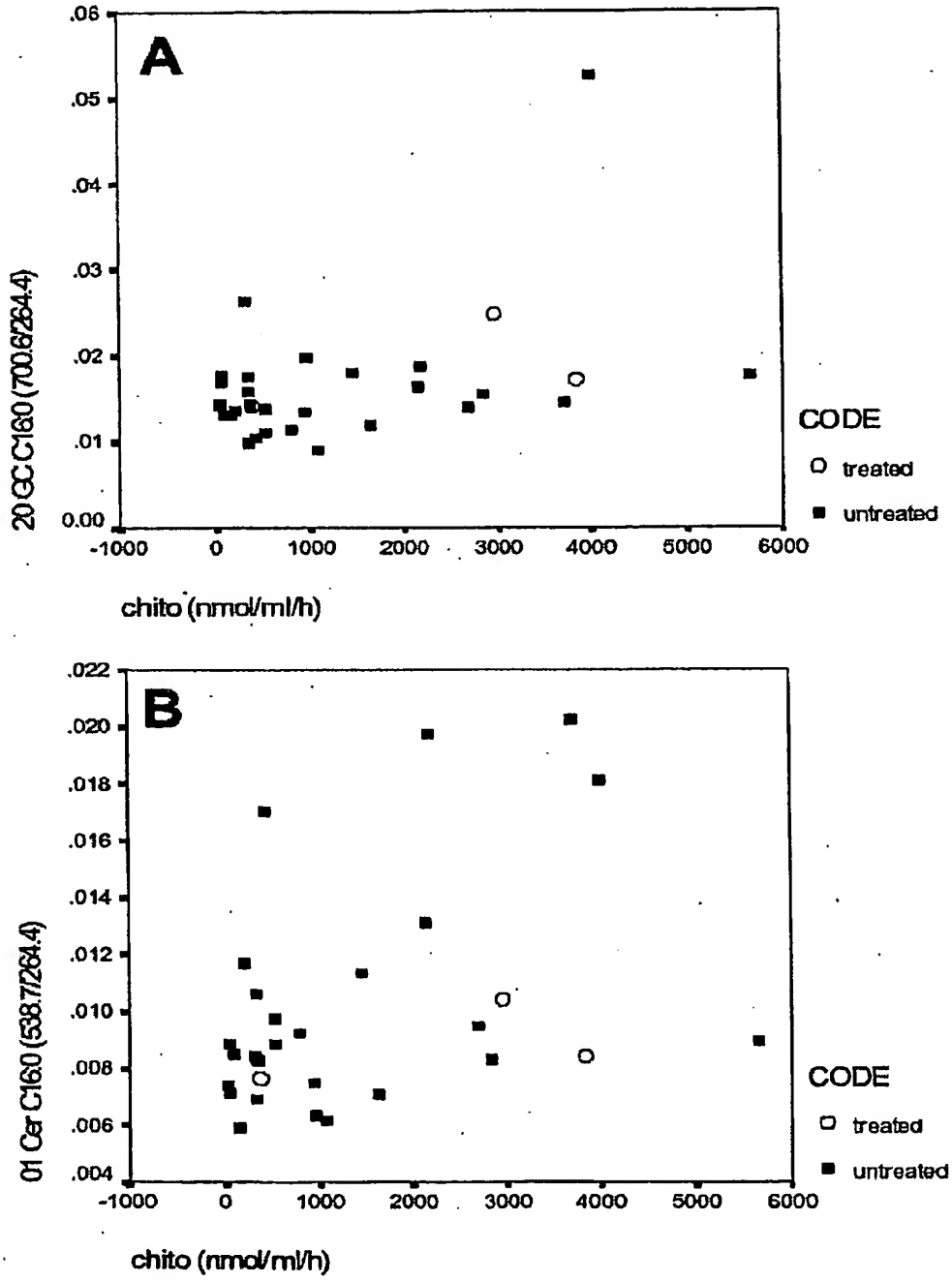

A
.06
.05
.04
.03
.02
.01
0.00
20 GC C160 (700.6/264.4)
-1000
0
1000
2000
3000
4000
5000
6000
chito (nmol/ml/h)
CODE
treated
untreated
B
.022
.020
.018
.016
.014
.012
.010
.008
.006
.004
01 Cer C16:0 (538.7/264.4)
-1000
0
1000
2000
3000
4000
5000
6000
chito (nmol/ml/h)
CODE
treated
untreated

FIGURE 7

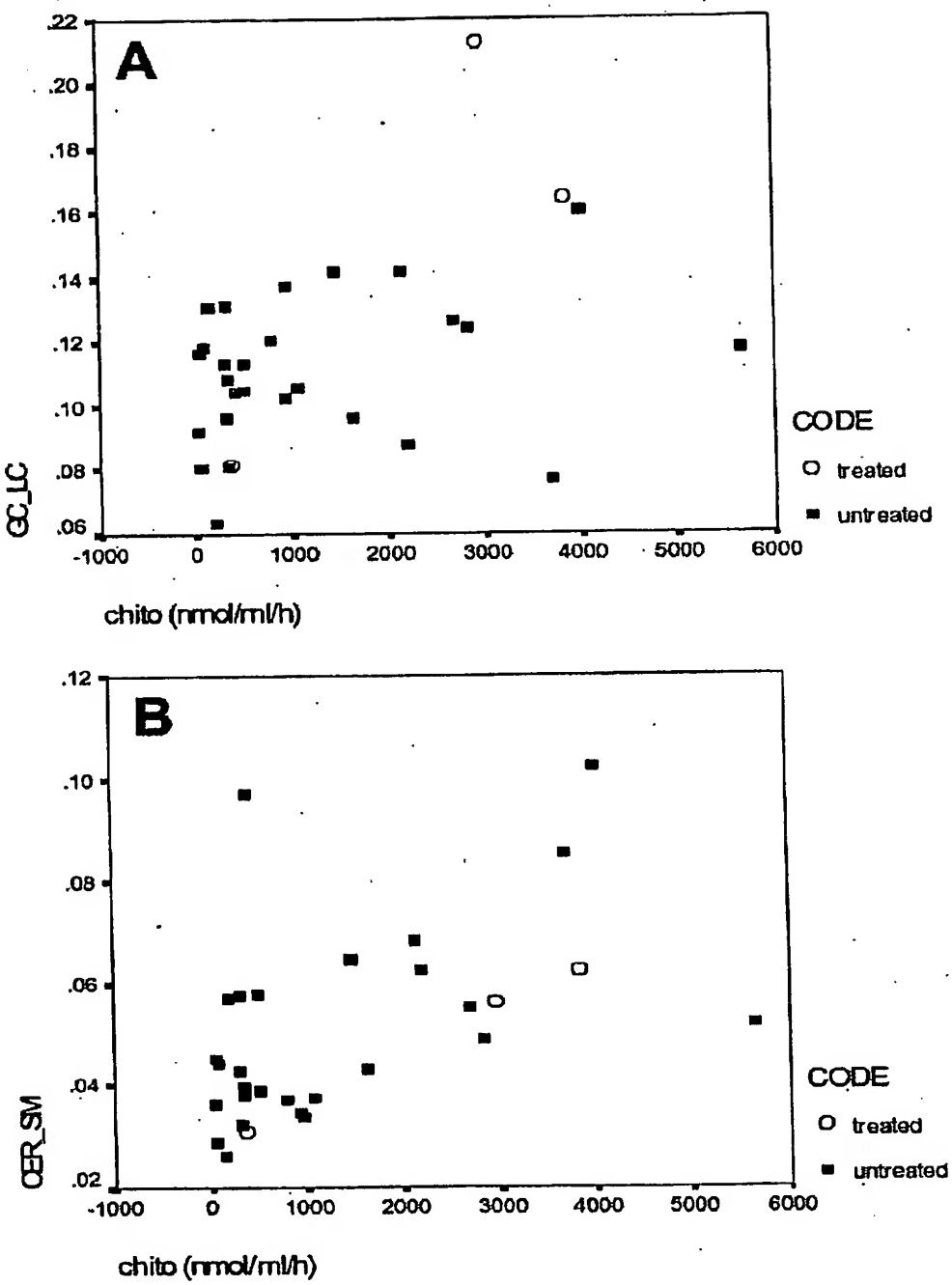
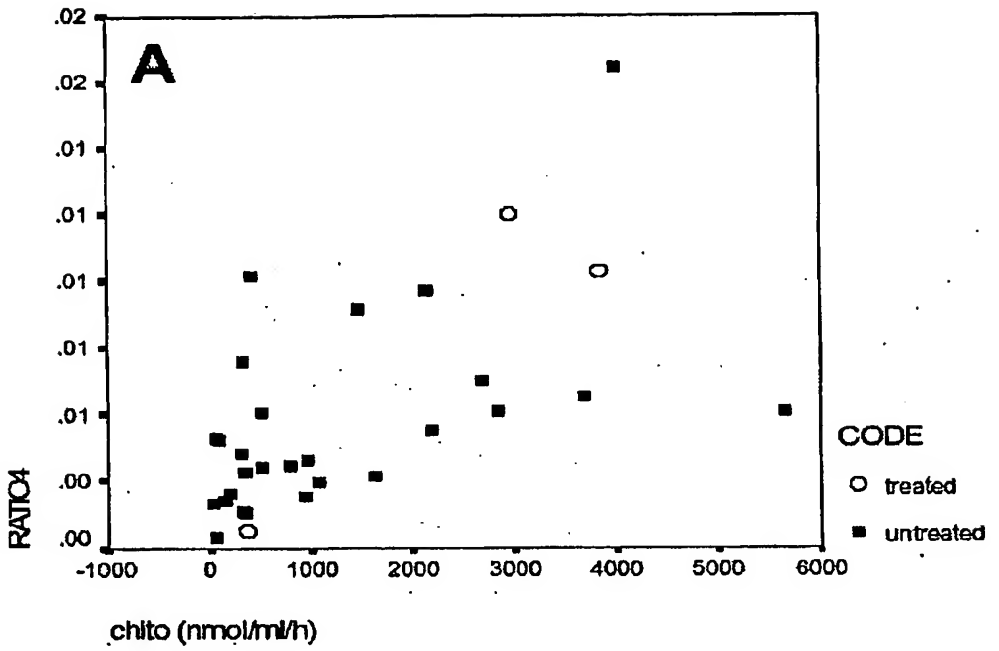

A
.22
.20
.18
.16
.14
.12
.10
.08
.06
GC_LC
-1000
0
1000
2000
3000
4000
5000
6000
chito (nmol/ml/h)
CODE
treated
untreated
B
.12
.10
.08
.06
.04
.02
CER_SM
-1000
0
1000
2000
3000
4000
5000
6000
chito (nmol/ml/h)
CODE
treated
untreated

FIGURE 8


A
.02
.02
.01
.01
.01
.01
.01
.00
.00
RATIO4
-1000
0
1000
2000
3000
4000
5000
6000
chito (nmol/ml/h)
CODE
treated
untreated

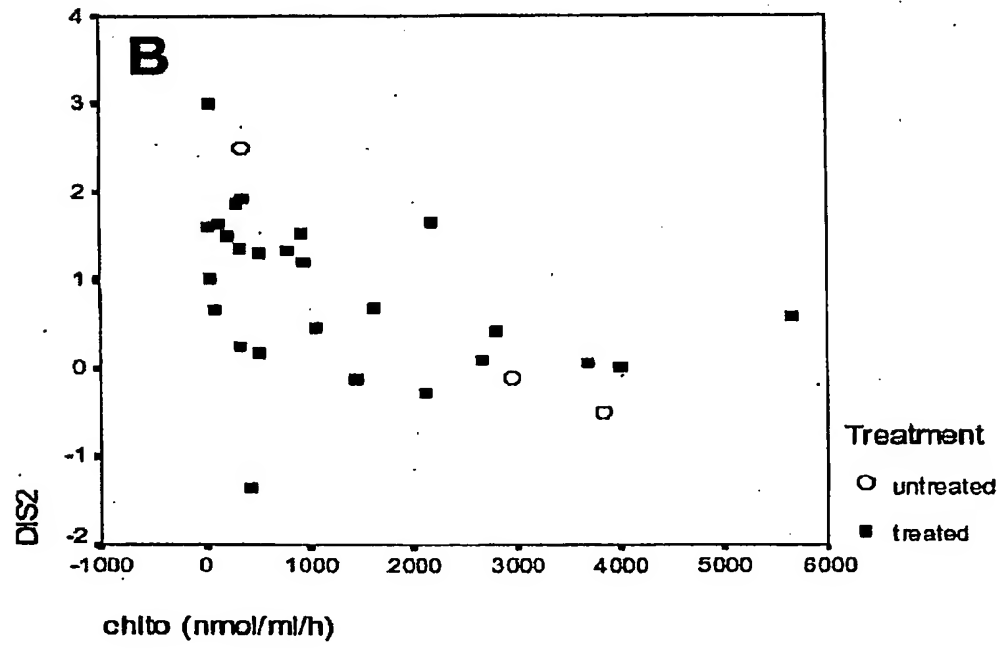

B
4
3
2
1
0
-1
-2
DIS2
-1000
0
1000
2000
3000
4000
5000
6000
chito (nmol/ml/h)
Treatment
untreated
treated

FIGURE 9

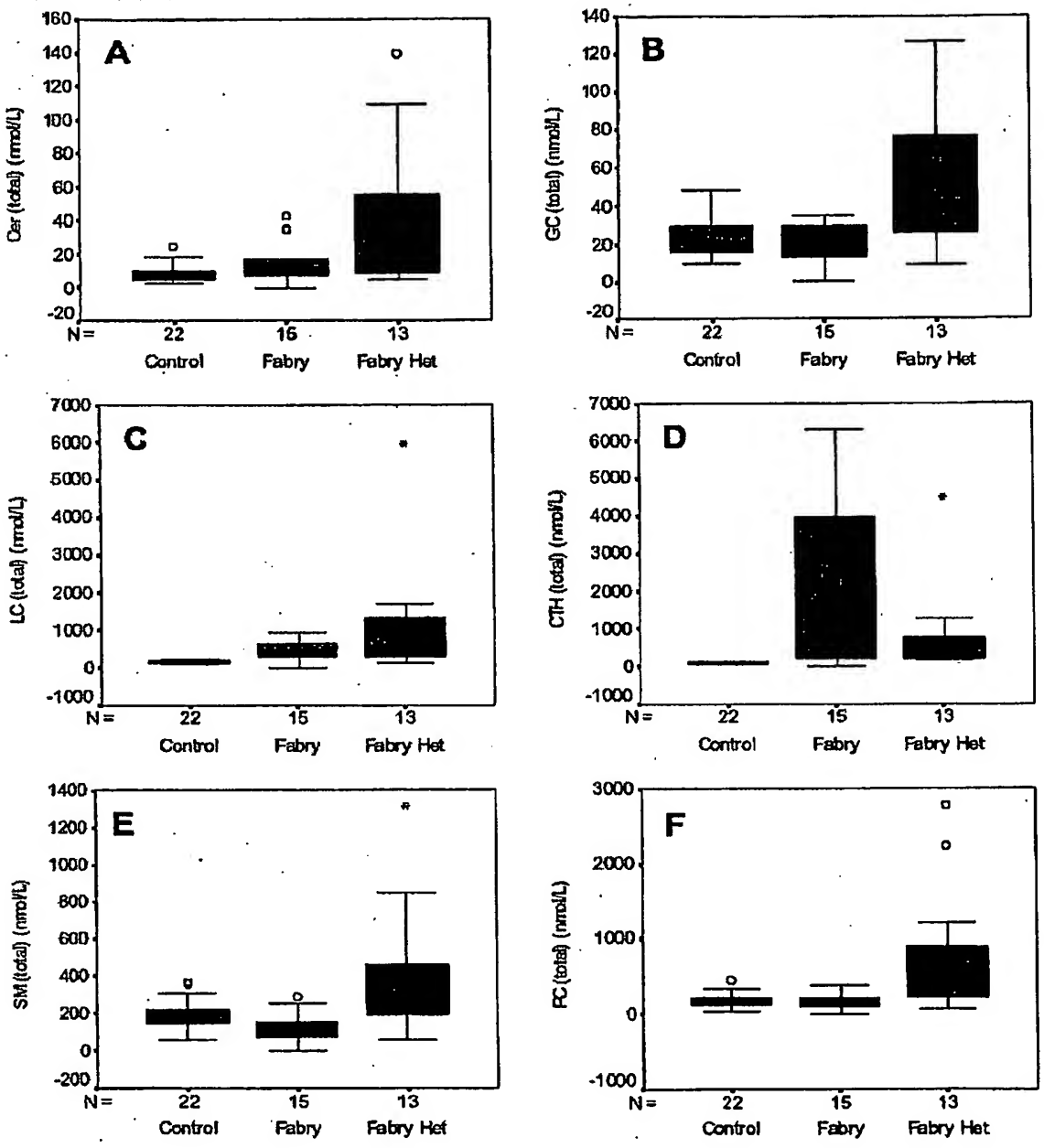

A
Cer (total) (nmol/L)
160
140
120
100
80
60
40
20
0
-20
N=
22
15
13
Control
Fabry
Fabry Het
B
GC (total) (nmol/L)
140
120
100
80
60
40
20
0
-20
N=
22
15
13
Control
Fabry
Fabry Het
C
LC (total) (nmol/L)
7000
6000
5000
4000
3000
2000
1000
0
-1000
N=
22
15
13
Control
Fabry
Fabry Het
D
CTH (total) (nmol/L)
7000
6000
5000
4000
3000
2000
1000
0
-1000
N=
22
15
13
Control
Fabry
Fabry Het
E
SM (total) (nmol/L)
1400
1200
1000
800
600
400
200
0
-200
N=
22
15
13
Control
Fabry
Fabry Het
F
PC (total) (nmol/L)
3000
2000
1000
0
-1000
N=
22
15
13
Control
Fabry
Fabry Het

FIGURE 10

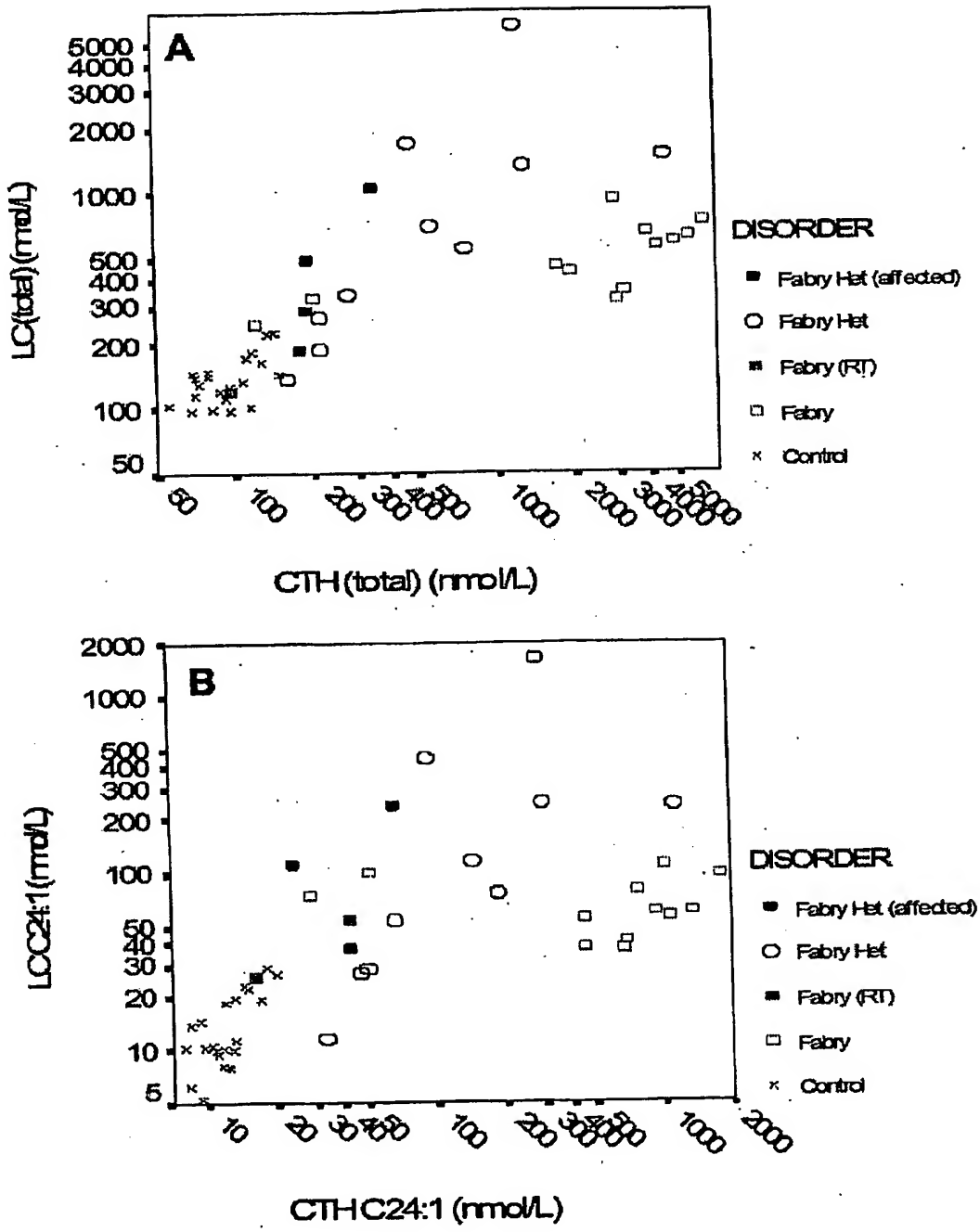

A
LC(total) (nmol/L)
5000
4000
3000
2000
1000
500
400
300
200
100
50
50
100
200
300
400
500
1000
2000
3000
4000
5000
CTH (total) (nmol/L)
DISORDER
Fabry Het (affected)
Fabry Het
Fabry (RT)
Fabry
Control
B
LCC24:1 (nmol/L)
2000
1000
500
400
300
200
100
50
40
30
20
10
5
10
20
30
40
50
100
200
300
400
500
1000
2000
CTHC24:1 (nmol/L)
DISORDER
Fabry Het (affected)
Fabry Het
Fabry (RT)
Fabry
Control

FIGURE 11

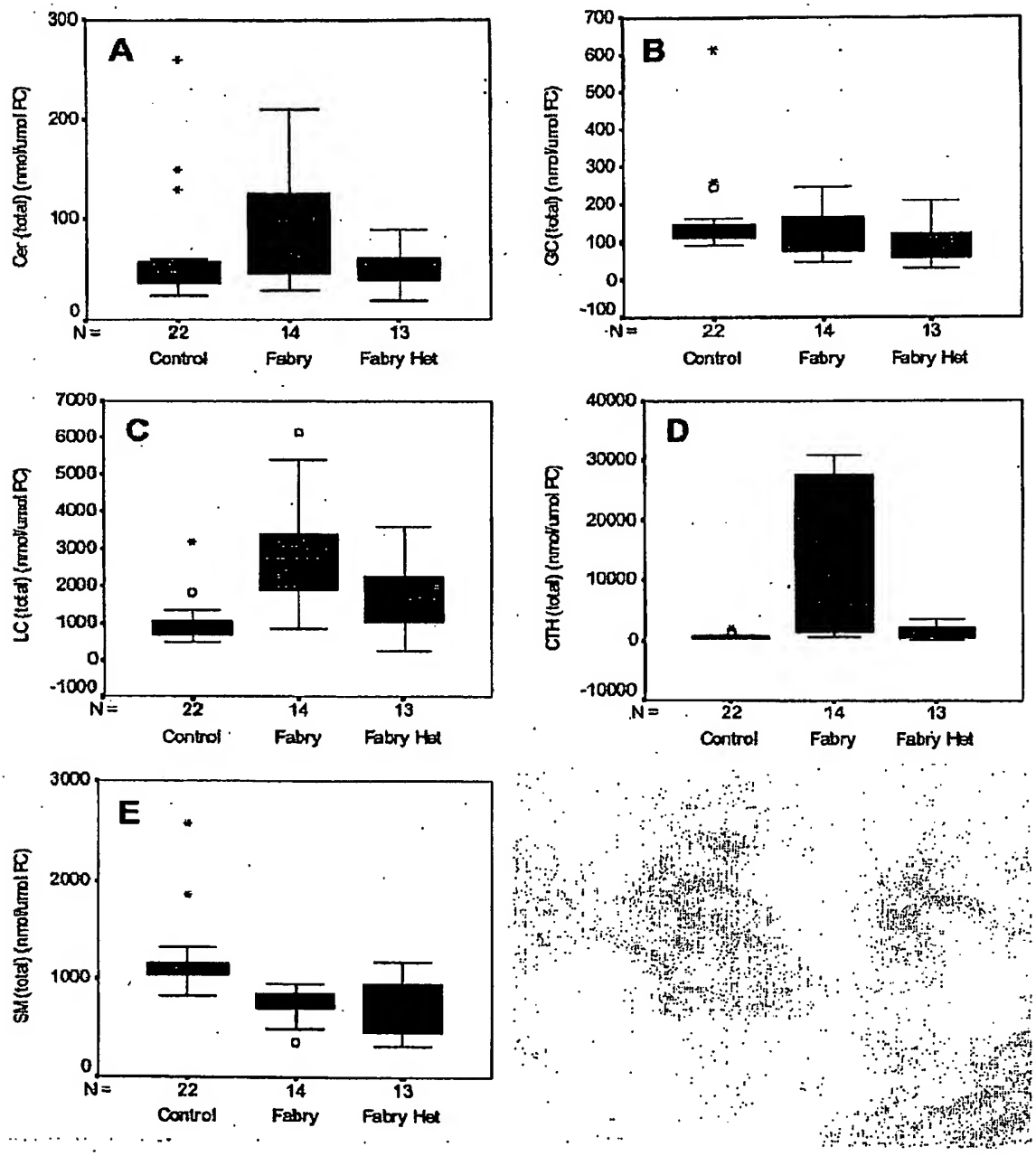

A
Cer (total) (nmol/umol PC)
300
200
100
0
N=
22
14
13
Control
Fabry
Fabry Het
B
GC (total) (nmol/umol PC)
700
600
500
400
300
200
100
0
-100
C
LC (total) (nmol/umol PC)
7000
6000
5000
4000
3000
2000
1000
0
-1000
D
CTH (total) (nmol/umol PC)
40000
30000
20000
10000
0
-10000
E
SM (total) (nmol/umol PC)
3000
2000
1000
0

FIGURE 12

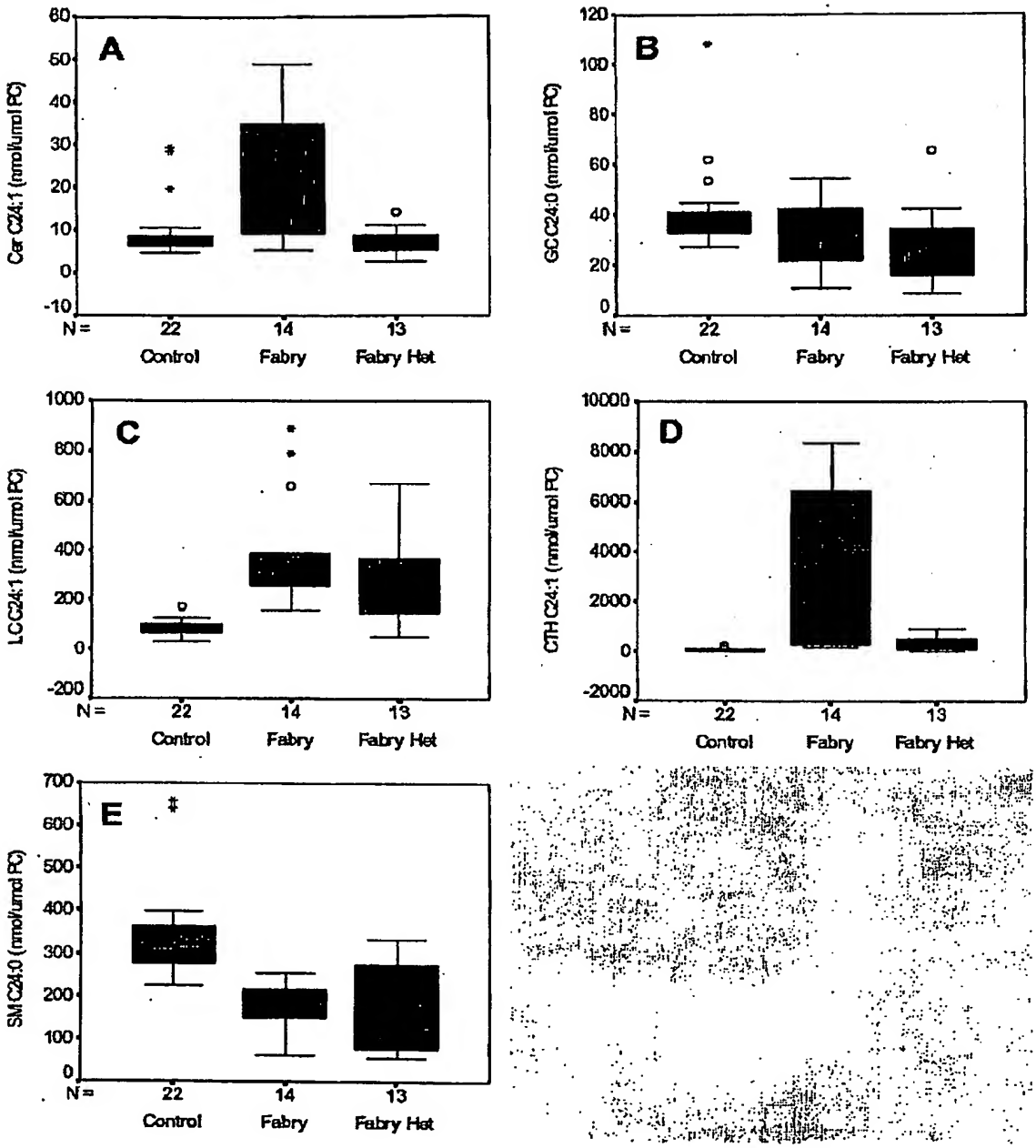

A
Car C24:1 (nmol/umol PC)
60
50
40
30
20
10
0
-10
N =
22
14
13
Control
Fabry
Fabry Het
B
GCC24:0 (nmol/umol PC)
120
100
80
60
40
20
0
C
LCC24:1 (nmol/umol PC)
1000
800
600
400
200
0
-200
D
CTH C24:1 (nmol/umol PC)
10000
8000
6000
4000
2000
0
-2000
E
SM C24:0 (nmol/umol PC)
700
600
500
400
300
200
100
0

FIGURE 13

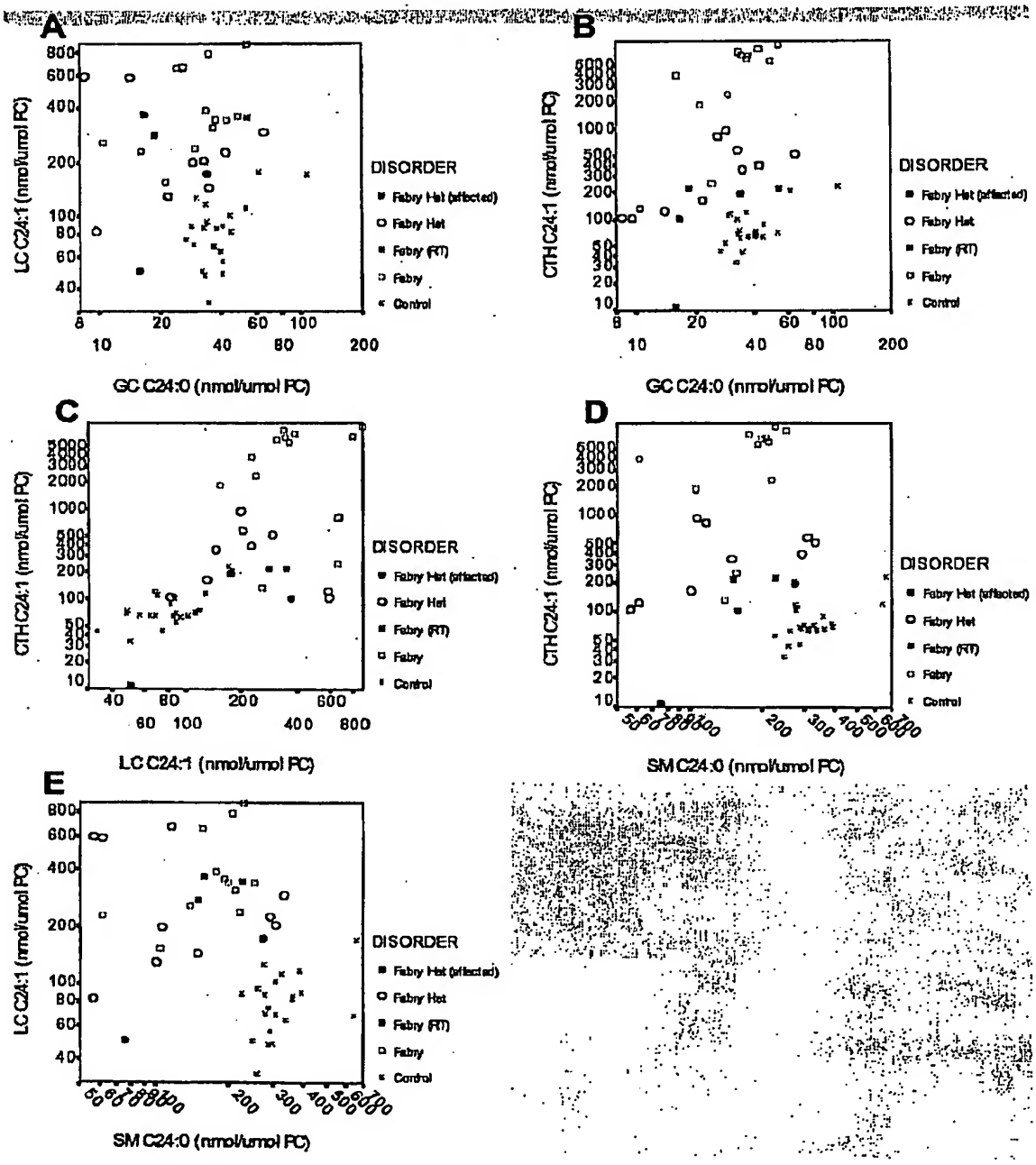

A
LC C24:1 (nmol/umol PC)
GC C24:0 (nmol/umol PC)
DISORDER
Fabry Het (affected)
Fabry Het
Fabry (RT)
Fabry
Control
B
CTHC24:1 (nmol/umol PC)
GC C24:0 (nmol/umol PC)
C
LC C24:1 (nmol/umol PC)
D
SM C24:0 (nmol/umol PC)
E
SM C24:0 (nmol/umol PC)

FIGURE 14

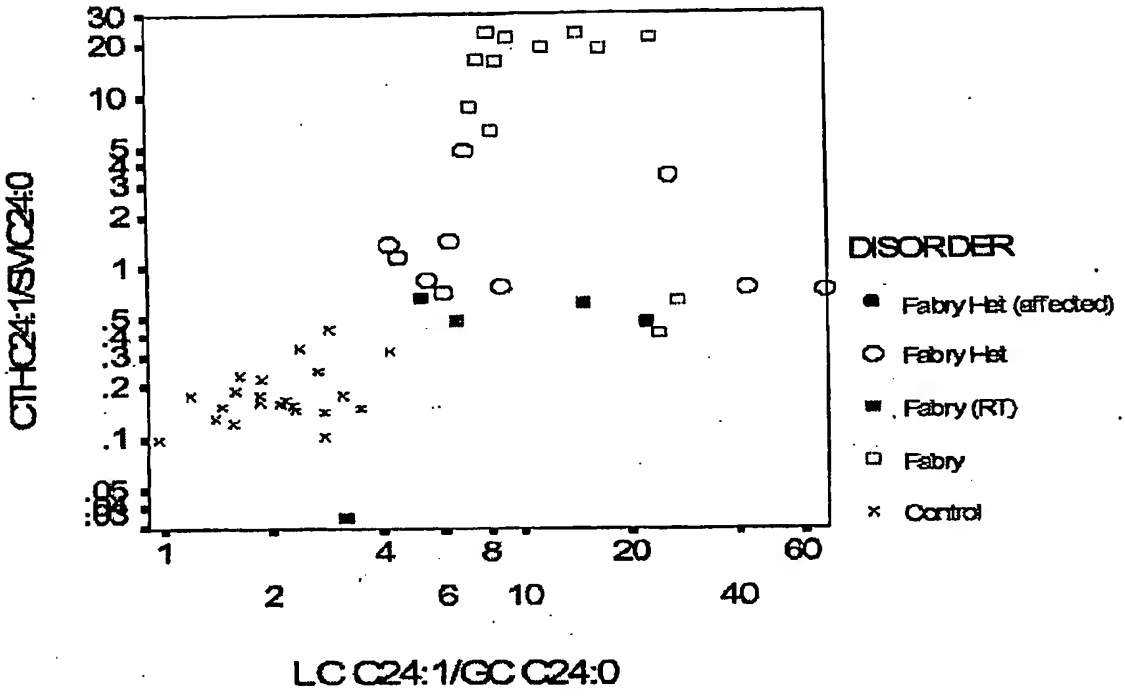

CTH-C24:1/SMC24:0
30
20
10
2
1
.1
1
2
4
6
8
10
20
40
60
LC C24:1/GC C24:0
DISORDER
Fabry Het (affected)
Fabry Het
Fabry (RT)
Fabry
Control

FIGURE 15

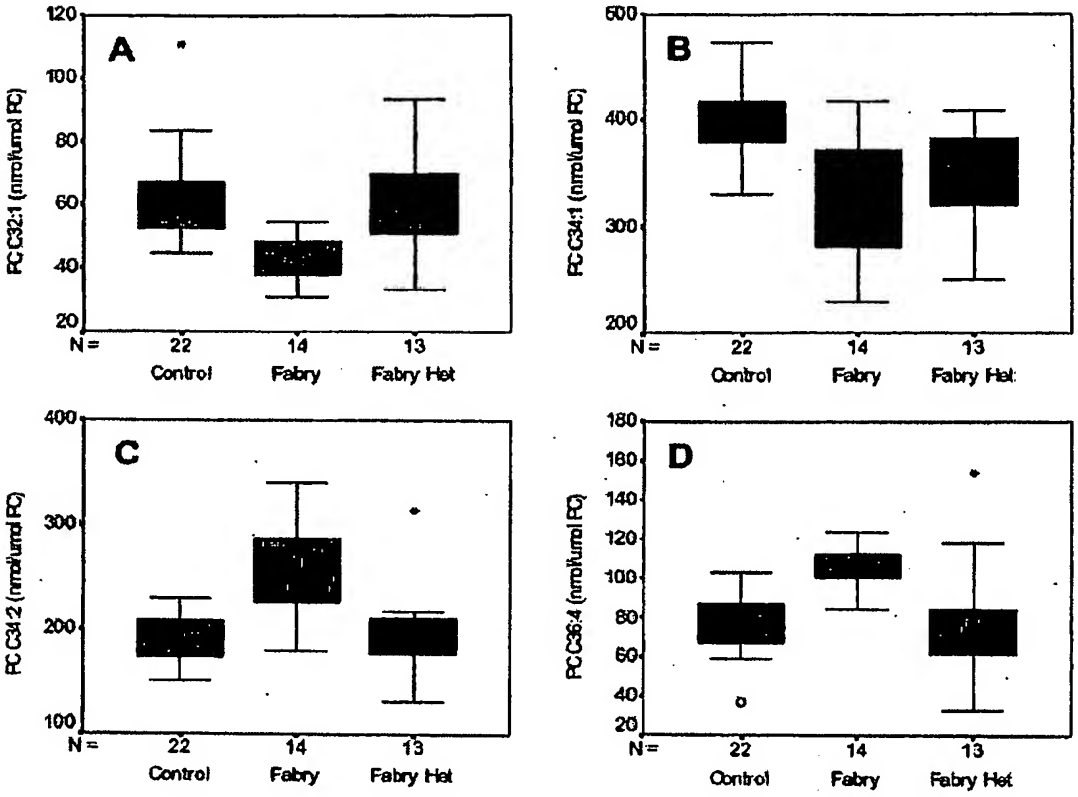

A
PC C32:1 (nmol/umol PC)
120
100
80
60
40
20
N =
22
14
13
Control
Fabry
Fabry Het
B
PC C34:1 (nmol/umol PC)
500
400
300
200
C
PC C34:2 (nmol/umol PC)
400
300
200
100
D
PC C36:4 (nmol/umol PC)
180
160
140
120
100
80
60
40
20

FIGURE 16

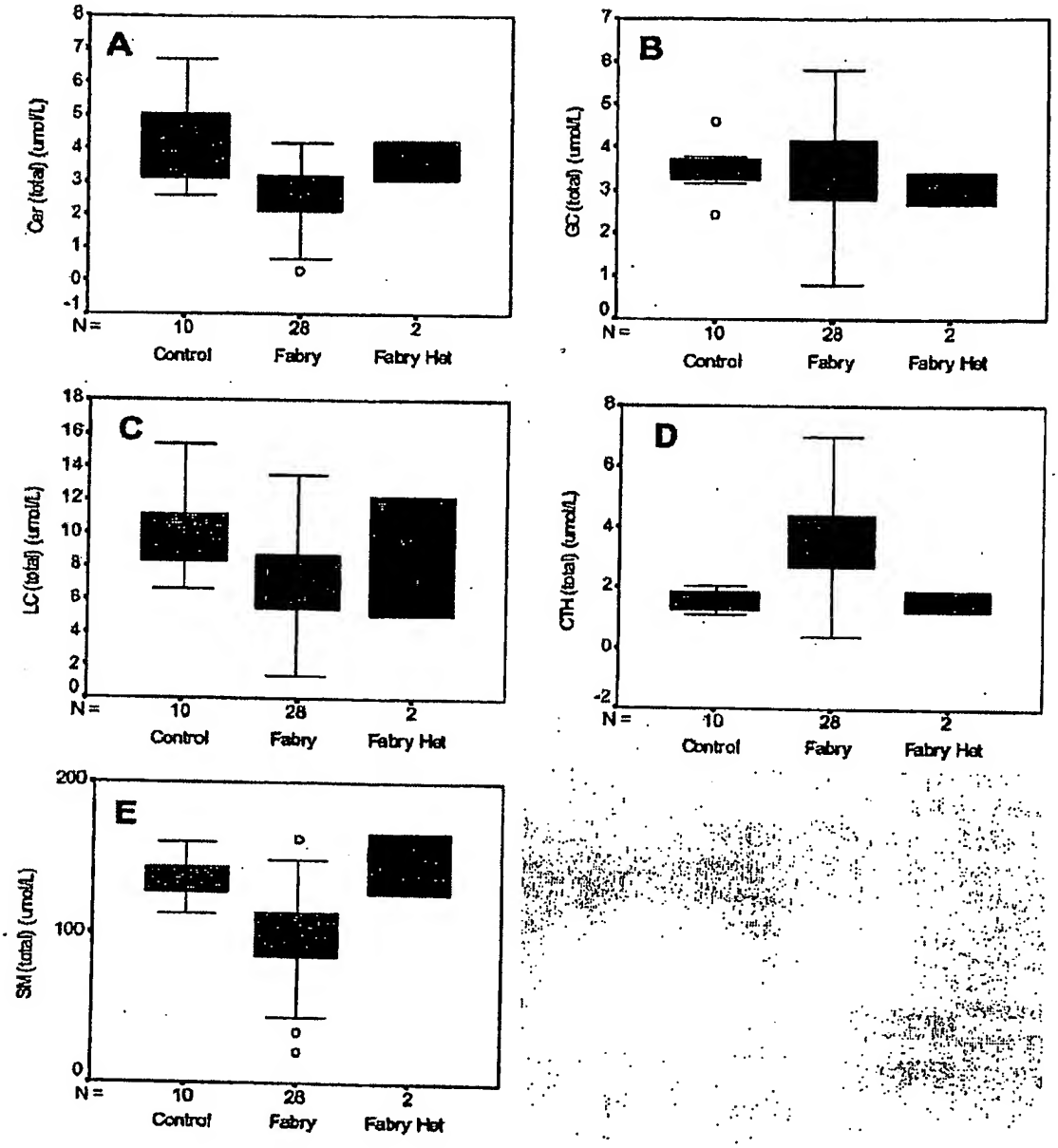

A
Cer (total) (umol/L)
N =
10
28
2
Control
Fabry
Fabry Het
B
GC (total) (umol/L)
C
LC (total) (umol/L)
D
CTH (total) (umol/L)
E
SM (total) (umol/L)

FIGURE 17

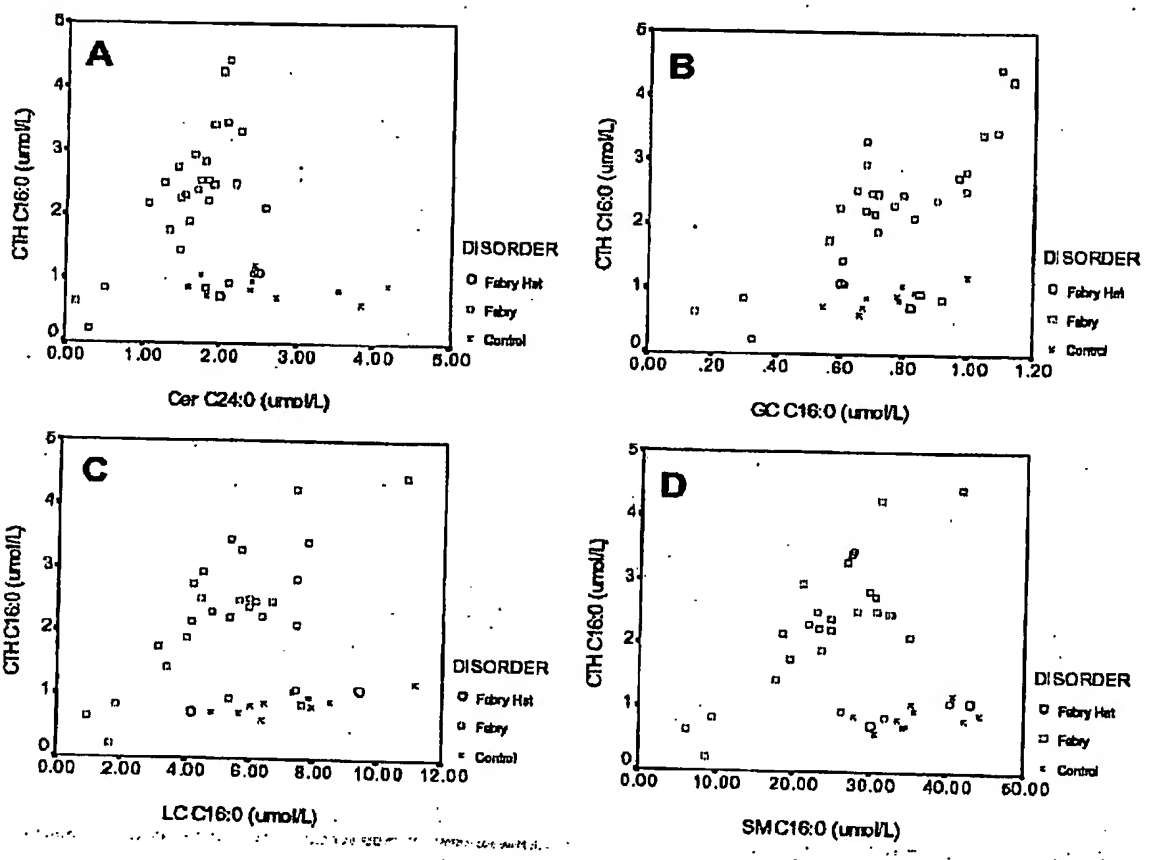

A
CTH C16:0 (umol/L)
Cer C24:0 (umol/L)
DISORDER
Fabry Het
Fabry
Control
B
CTH C16:0 (umol/L)
GC C16:0 (umol/L)
DISORDER
Fabry Het
Fabry
Control
C
CTH C16:0 (umol/L)
LC C16:0 (umol/L)
DISORDER
Fabry Het
Fabry
Control
D
CTH C16:0 (umol/L)
SM C16:0 (umol/L)
DISORDER
Fabry Het
Fabry
Control

FIGURE 18

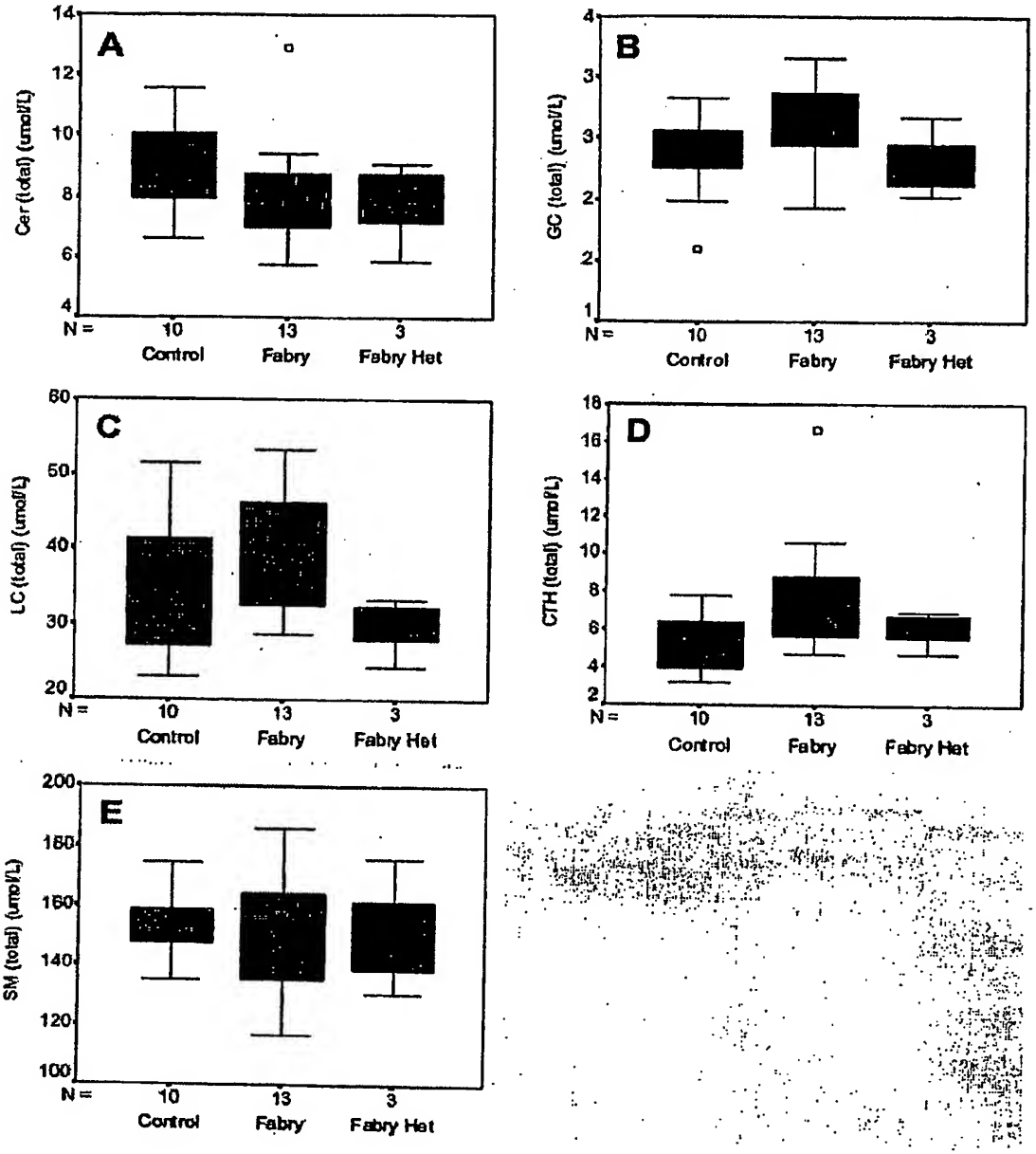

A
Cer (total) (umol/L)
14
12
10
8
6
4
N =
10
13
3
Control
Fabry
Fabry Het
B
GC (total) (umol/L)
4
3
2
1
C
LC (total) (umol/L)
60
50
40
30
20
D
CTH (total) (umol/L)
18
16
14
12
10
8
6
4
2
E
SM (total) (umol/L)
200
180
160
140
120
100

FIGURE 19

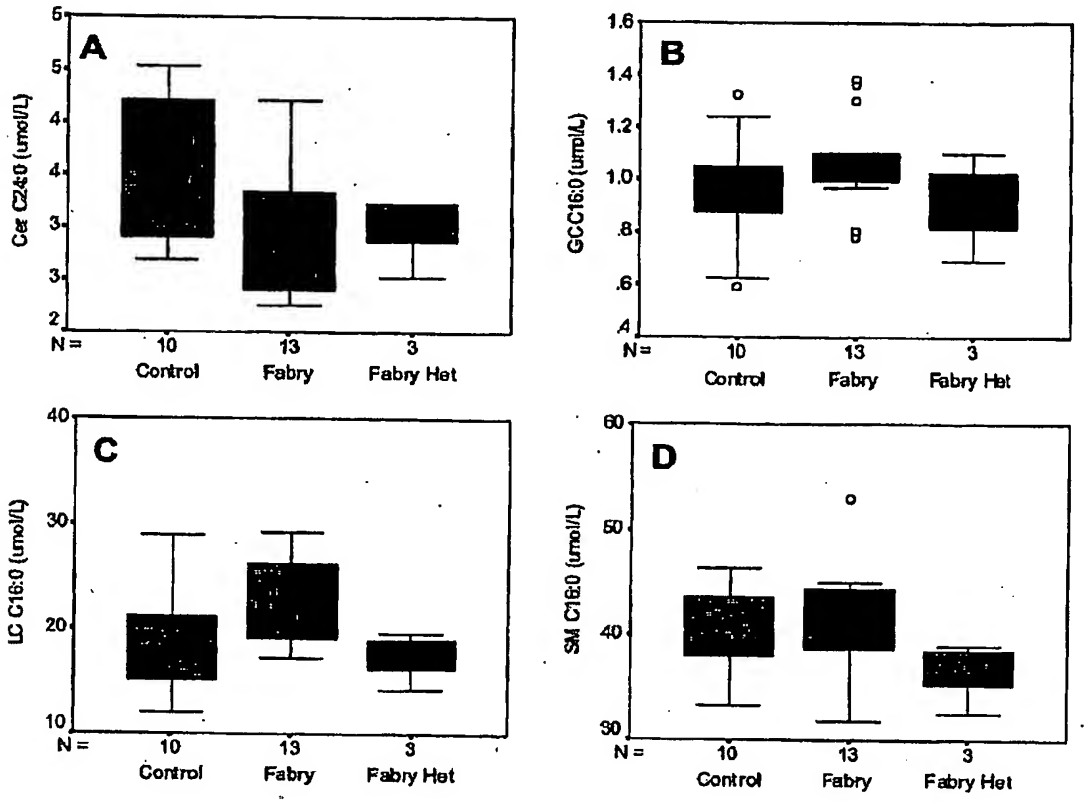

A
Cer C24:0 (umol/L)
N =
10
13
3
Control
Fabry
Fabry Het
B
GCC16:0 (umol/L)
C
LC C16:0 (umol/L)
D
SM C16:0 (umol/L)

FIGURE 20

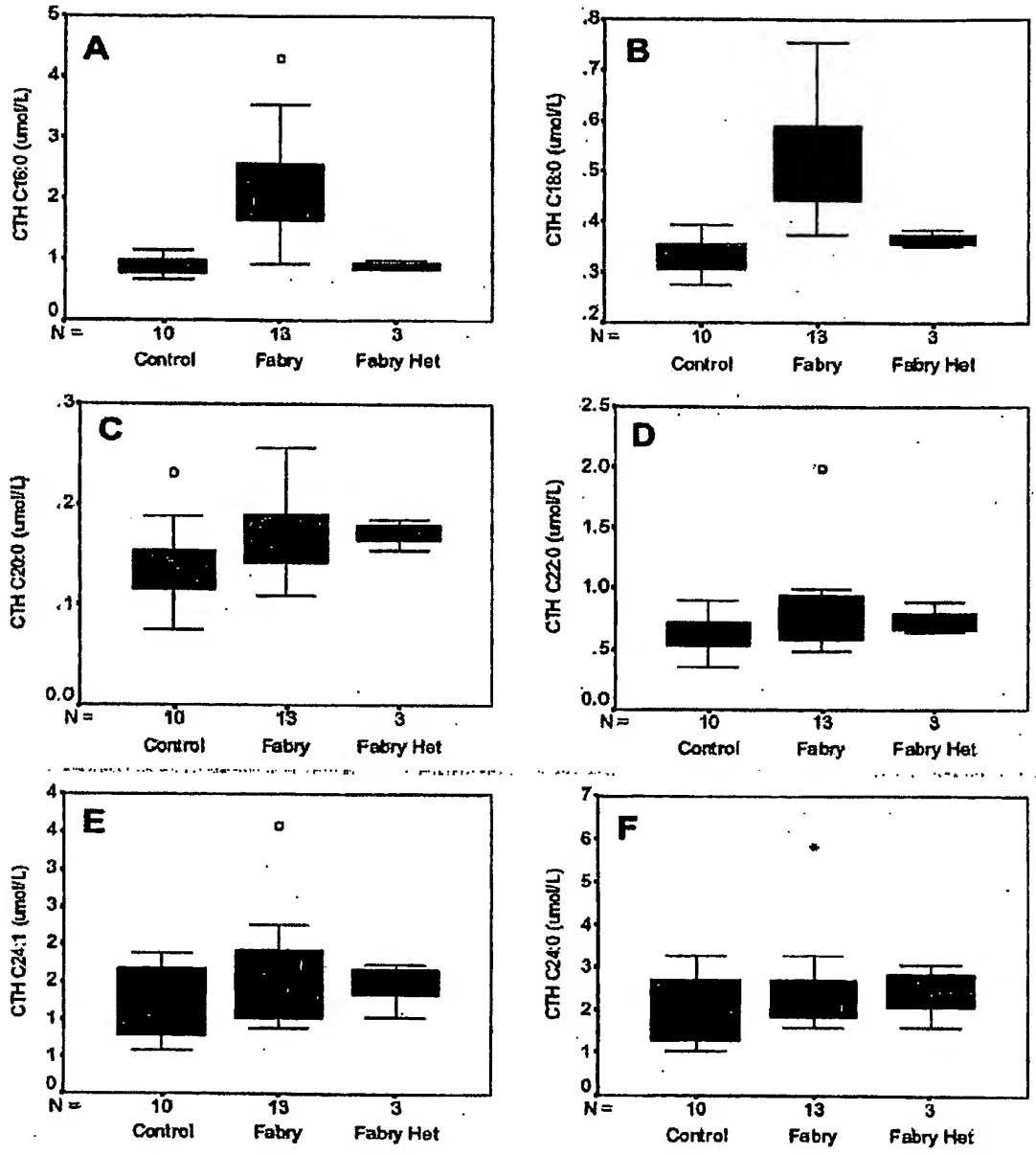

A
CTH C16:0 (umol/L)
N =
10
13
3
Control
Fabry
Fabry Het
B
CTH C18:0 (umol/L)
C
CTH C20:0 (umol/L)
D
CTH C22:0 (umol/L)
E
CTH C24:1 (umol/L)
F
CTH C24:0 (umol/L)

FIGURE 21

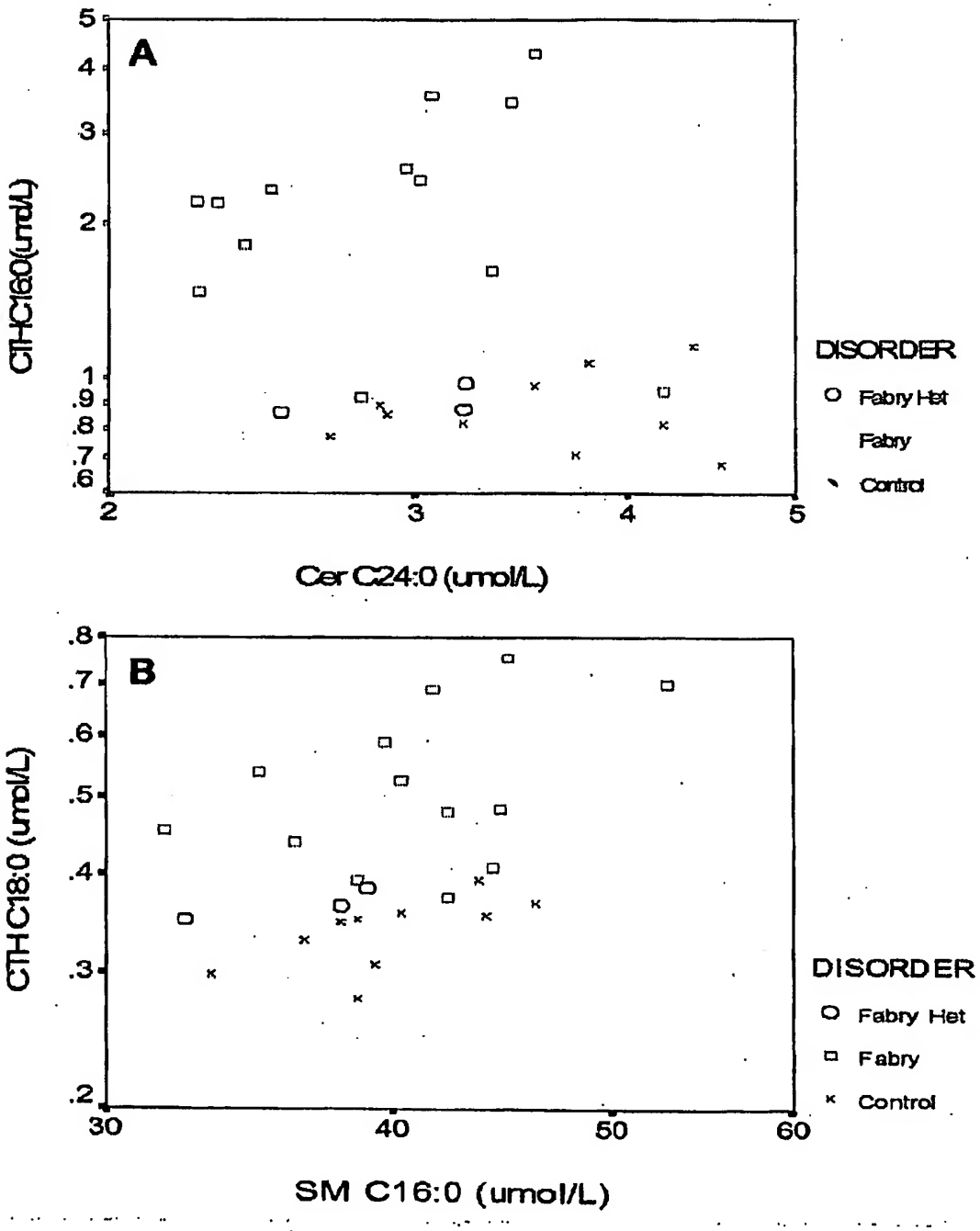

A
CTHC16O(umol/L)
5
4
3
2
1
.9
.8
.7
.6
2
3
4
5
Cer C24:0 (umol/L)
DISORDER
Fabry Het
Fabry
Control
B
CTH C18:0 (umol/L)
.8
.7
.6
.5
.4
.3
.2
30
40
50
60
SM C16:0 (umol/L)
DISORDER
Fabry Het
Fabry
Control

FIGURE 22